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Identification of Cumulative Assessment Groups of Pesticides [1]

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Abstract

The objective of the project was to identify common assessment groups (CAGs) for the pesticide active substances included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009). Initially, 120 substances were excluded for further consideration using objective criteria. For the remaining 224 substances information regarding e.g. name, chemical identity, pesticidal mode of action, and toxicological effects was collected. The establishment of CAGs was based on a tiered approach comprising up to four CAG levels spanning from grouping of all compounds having effects on a given target organ or tissue at CAG Level 1 to grouping at CAG Level 4 only those compounds for which a common mechanism of action could been established. Draft Assessment Reports (DARs) prepared for the "European Commission Programme for inclusion of Active Substances in Annex I of Council Directive 91/414/EEC (Articles 5 and 6 of Council Directive 91/414/EEC)" were used to identify the relevant target organs and tissues (end-points) for the toxicological effects of the active substances. For each relevant target all compounds exerting a toxicological effect were allocated to CAG Level 1. In the next step detailed toxicological information was scrutinized and compounds showing a common toxic effect on a phenomenological/specific effect basis in each target organ and tissue were grouped into CAGs at Level 2. Refined CAGs were established when it could be demonstrated that the compounds actually possess the same mode of action (CAG Level 3) or mechanism of action (CAG Level 4). All chemical and toxicological information that formed the basis for the establishment of CAGs was included in a searchable database.

Summary

EFSA has initiated a project to provide a report that identifies the toxicological effects and endpoints that can form the basis for allocating the active pesticide substances included in Annex I. of Council Directive 91/414/EEC (up to 31st of May 2009) into common assessment groups (CAG) and to propose specific CAGs of the pesticides for consideration in MRL setting. In addition, a searchable database should be prepared for the effects identified as important for cumulative risk assessment listing the pesticide active substances having these effects with the related endpoints together with their no observed adverse effect levels (NOAEL).

Regulation of the European Council and the European Parliament (EC) No. 396/2005 on Maximum Residue Levels (MRLs) for pesticides in food emphasises the importance "to carry out further work to develop a methodology to take into account cumulative and synergistic effects of pesticides". As there were no internationally agreed methodology to assess risks from simultaneous exposure to more than one pesticide, EFSA in November 2006, started to work on cumulative risk assessment of pesticides. In opinions of EFSA's Scientific Panel on Plant Protection Products and their Residues (PPR Panel) on how to assess cumulative risk from pesticides to human health the PPR Panel proposed to follow a step-wise approach to identify a common assessment group (CAG) consisting of pesticides having a common mode of action.

Experimental studies have produced strong evidence that mixtures of chemicals with common specific modes of action work together to produce combined effects that are larger than the effects of each mixture component applied singly. The principle of dose additivity apply for

such chemicals and mean that mixture effects are to be expected even when each chemical is present below zero-effect levels, because it is assumed that all toxicants in the mixture behave as if they were a dilution of one another. Hence, any concentration of any compound needs to be considered because it may add to the mixture concentration.

Initially, 120 substances were excluded for further consideration using objective criteria. For the remaining 224 substances information regarding e.g. name, chemical identity, pesticidal mode of action, and toxicological effects was collected. Information on toxicological effects and potential modes and/or mechanisms of action for mammalian toxicity were obtain from Draft Assessment Reports (DARs), DAR addenda, and EFSA/ECCO/EPCO peer review reports, reports from the Joint FAO/WHO Meting on Pesticide Residues (JMPR), and the open literature.

The establishment of CAGs was based on a tiered approach comprising up to four CAG levels spanning from grouping of all compounds having effects on a given target organ or tissue at CAG Level 1 to grouping of only those compounds at CAG Level 4 for which a common mechanism of action could been established. Draft Assessment Reports (DARs) prepared for the "European Commission Programme for inclusion of Active Substances in Annex I of Council Directive 91/414/EEC (Articles 5 and 6 of Council Directive 91/414/EEC)" were used to identify the relevant target organs and tissues (end-points) for the toxicological effects of the active substances. For each relevant organ/tissue all compounds excerting a toxicological effect were allocated to CAG Level 1. In the next step detailed toxicological information was scrutinized and compounds showing a common toxic effect on a phenomenological/specific effect basis in each target organ and tissue were grouped into CAGs at Level 2. Refined CAGs were established when it could be demonstrated that the compounds actually possess the same mode of action (CAG Level 3) or mechanism of action (CAG Level 4).

All chemical and toxicological information that formed the basis for the CAGs was included in an Excel spreadsheet containing the following entries: Name of active substance, pesticiddal category, chemical class, IUPAC name, CAS number, pesticidal mode/mechanism of action, ADI, ArfD, AOEL, target organ/tissue (CAG level 1), phenomenological / specific effect (CAG level 2), species, strain, sex, study duration, route of exposure, NOAEL, LOAEL, reference, source (DAR, EFSA peer-review, open literature, etc.), remarks, toxicological mode of action (CAG level 3), and toxicological mechanism of action (CAG level 4).

A searchable database in Microsoft Access was established based on the Excel spreadsheet.

For the following target organs/tissues, recommendations are given on potential CAGs that should be considered for use in cumulative risk assessment (CRA):

- Adrenal gland (chapter 7)
- Bone marrow (chapter 8)
- Bones / skeleton (chapter 9)
- Cardiovascular system (chapter 10)
- Eye (chapter 11)
- Gallbladder (chapter 12)

- Haematological system (chapter 14)
- Kidney (chapter 16)
- Liver (chapter 17)
- Muscles (chapter 20)
- Nervous system (chapter 21)
- Parathyroid gland (chapter 23)
- Reproductive system and developmental toxicity (chapter 25)
- Spleen (chapter 28)
- Thyroid gland (chapter 30)
- Urinary bladder (chapter 31)

No CAGs were recommended for CRA the following target organs/tissues:

- Gastrointestinal tract (chapter 13)
- Immune system (chapter 15)
- Lung (chapter 18)
- Lymph node (chapter 19)
- Pancreas (chapter 22)
- Pituitary gland (chapter 24)
- Salivary gland (chapter 26)
- Skin (chapter 27)
- Thymus (chapter 29)

In conclusion, CAGs at level 3 based on knowledge on the mode of action could only be recommended for the bone marrow, bones / skeleton, eye, haematological system, liver, muscles, nervous system, reproductive and developmental system, and thyroid gland, whereas CAGs at level 4 based on the mechanism of action could only be recommended for some toxicological effects on the eye, liver, nervous system and reproductive and developmental system. These CAGs should provide a reasonable basis for performing cumulative risk assessments (CRA).

The CAGs at level 2 are established without any knowledge about mode/mechanism of action and do therefore not fulfil the criteria for a cumulative mechanism group that can be expected to exert dose additivity. However, risk managers may wish to use a CAG at level 2 in order to consider whether a realistic cumulative exposure to certain active substances would need to be further investigated.

Data on mode/mechanism of action are lacking for the majority of the different toxicological effects of the active pesticide substances included in the assessment. However, before using expensive resources to obtain such information a systematic review of pesticide occurrence and exposure data for food in Europe should be carried out with the aim of identifying combinations of active pesticide substances that are either realistic candidates or not for cumulative risk assessment.

Although the CAGs at level 2 do not fulfil the criteria for a cumulative mechanism group that can be expected to exert dose additivity they can be useful for a preliminary assessment of realistic mixtures of active substances. This in particular, because it can be expected that such an assessment will be conservative in the absence of dose additivity between the compounds.

For such an assessment, it is recommended to use the Reference point index (RfPI) (based on the NOAELs for the compounds in the CAG) as described by the PPR Panel in 2008. The RfPI is similar to the Point of departure index (PODI) that was advocated by EFSA in 2007 and by WHO in 2009 to be used in cumulative risk assessment.

Key words:

Cumulative risk assessment, cumulative assessment group, mechanism of action, mode of action, phenomenological effects, pesticidal active substance.

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Background

Regulation of the European Council and the European Parliament (EC) No. 396/2005 on Maximum Residue Levels (MRLs) for pesticides in food emphasises the importance "to carry out further work to develop a methodology to take into account cumulative and synergistic effects of pesticides". As there were no internationally agreed methodology to assess risks from simultaneous exposure to more than one pesticide, EFSA in November 2006, started to work on cumulative risk assessment of pesticides by organising a colloquium on "Cumulative risk assessment of pesticides to human health: the way forward". The summary report of this colloquium includes the results from two discussion groups dealing with cumulative exposure (EFSA 2007).

In 2008, EFSA's Scientific Panel on Plant Protection Products and their Residues (PPR Panel) elaborated an opinion "to evaluate the suitability of existing methodologies and, if appropriate, the identification of new approaches to assess cumulative and synergistic risk from pesticides to human health with a view to set MRLs for those pesticides in the frame of Regulation (EC) No. 396/2005" (EFSA 2008). The PPR Panel proposed to follow a step-wise approach to identify a common assessment group (CAG) consisting of pesticides having a common mode of action. The proposal was mainly based on US EPA documents (EPA, 1999; 2002) and an ILSI report (ILSI, 1999). According to EPA (1999) a "Common Mechanism Group (CMG) consists of chemicals for which scientifically reliable data demonstrate that the same toxic effect occurs in or at the same organ or tissue by essentially the same sequence of major biochemical events. However, not all members of a CMG should necessarily be included in a more refined quantitative estimate of cumulative risk".

The PPR Panel agreed that full consideration of all of the criteria put forward for grouping compounds in CAGs would provide the most sound and robust grouping. However, for the purposes of risk assessment, compounds might be grouped even in the absence of such detailed data, on the basis of a less refined evaluation of the mode of action (i.e., based only on target organ toxicity). (EFSA, 2008).

Following the general opinion of the PPR Panel, the proposed tiered approach to assess cumulative effects from exposure to pesticides was tested for a selected group of triazole pesticides (EFSA 2009). The PPR Panel concluded that the previously proposed tiered approach could be simplified by starting with a CAG as refined as the data allow and using the same CAG in all steps of the assessment, and restricting the exposure scenario to two tiers, one deterministic and one probabilistic tier.

Overall, the PPR Panel concluded that although a tiered approach is an appropriate way to address cumulative dietary risk assessment it cannot yet be applied on a routine basis. First, the following issues should be resolved:

- The basis for and establishment of relevant CAGs, on an European level
- Confirmation that both the deterministic and probabilistic approaches for cumulative exposure assessment provide appropriate levels of protection
- Completion of further guidance on appropriate methodologies for exposure assessment.

Therefore EFSA launched a call to obtain proposals for a project, which will search and explore the existing pesticide data bases, open literature and Draft Assessment Reports (DARs) to identify the toxicological effects and endpoints that can be the basis of a cumulative risk assessment. Furthermore proposal for common assessment groups of active substances having these identified effects and the related endpoints should be made. The aim of this project is to set the basis for carrying out of cumulative risk assessments on a routine basis in MRL setting.

Terms of reference

The specifications of the invitation to tender defined the following tasks and these are taken as the term of reference for this report.

Scrutinize the available databases and DARs of pesticides included to Annex I. of Council Directive 91/414/EEC (up to 31st of May 2009) in order to identify every single specific effects and their respective endpoints that can be the basis of a Common Assessment Group on which a cumulative risk assessment should be performed. To the best possible extent, assumption of the existence of specific mode of action (MOA) will have to be substantiated by identifying appropriate information in open scientific literature.

Collect NOAELs and mechanistic data for the identified effects, if available or sufficiently covered in the peer reviewed dossier prepared in line with Council Directive 91/414/EEC as well as resulting from the evaluation of scientific literature.

Develop the definition of non specific effects and establishment of criteria allowing concluding that an effect meets the definition of non specific effect. List and discuss non-specific effects that will be considered not relevant for cumulative risk assessment.

Prepare a searchable database of the effects identified as important for cumulative risk assessment, and the list of pesticide active substances having these effects with the related endpoints.

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Introduction and Objectives

INTRODUCTION

During more than two decades several suggestions have been published on how to perform risk assessment on mixtures of pesticides (reviewed by Kortenkamp et al. 2009; Reffstrup et al. 2010). In 1986 the Environmental Protection Agency in USA (US EPA) published a guideline for health risk assessment of chemical mixtures (EPA, 1986). However, what really put focus on this topic was the Food Quality Protection Act of 1996 which concerning pesticide residues requires US EPA to consider ”available information concerning the cumulative effects of such residues and other substances that have a common mechanism of toxicity” (United States of America in Congress, 1996). Since then US EPA has published several reports and guidelines on health risk assessment of chemical mixtures (EPA, 1999a, 2000, 2002, 2003).

The Agency for Toxic Substances and Disease Registry in USA (ATSDR) has published two guidelines with instructions to users on how to apply current methodologies for risk assessment of combined actions of chemicals (ATSDR, 2001, 2004).

In 2002 the Health Council of The Netherlands as well as the Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment in United Kingdom published advisory reports on risk assessment of mixtures (COT, 2002; Feron et al., 2004; Health Council of the Netherlands, 2002).

The Danish Veterinary and Food Administration has published the reports “Combined Actions of Pesticides in Food” (Reffstrup, 2002) and “Combined Actions and Interactions of Chemicals in Mixtures” (Binderup et al., 2003) which summarised and evaluated the present knowledge about combined toxic effects of mixtures of chemicals.

Since then, international initiatives have been taken in order to more closely explore what approaches can be used to evaluate chemical mixtures. Most notably, the European Food Safety Authority (EFSA) organised a workshop on cumulative risk assessment of pesticides (EFSA, 2007) and the Norwegian Scientific Committee for Food Safety and EFSA have published opinions on risk assessment of combined actions on chemicals (EFSA, 2008; Norwegian Scientific Committee for Food Safety (VKM 2008). In addition, WHO/IPCS hosted an International Workshop on Aggregate/Cumulative Risk Assessment in Washington in March 2007 (IPCS 2009a).

Based on all these reports, there is strong evidence that chemicals with common specific modes of action work together to produce combined effects that are larger than the effects of each mixture component applied singly. The literature shows that this applies to a number of different endpoints of relevance to mammalian toxicology and there is a consensus in the field of mixture toxicology that the customary chemical-by chemical approach to risk assessment might be too simplistic (Kortenkamp et al. 2009).

The basic types of combined action of compounds are either combined effect without interaction in the form of simple similar action (dose addition) and simple dissimilar action (response addition) or combined effect with interaction (antagonism, synergism). Many terms have been used for additivity, but it seems as the terminology that has become fairly common

includes the terms simple similar action and simple dissimilar action to describe additivity (Teuschler, 2007).

The model for simple similar action (synonyms: dose additivity, Loewe additivity) assumes that the compounds in the mixture behave as if they are dilutions of each other. This means that the compounds act on the same biological site by the same mechanism/mode of action and differ only in their potencies. The theoretical basis for the simple dissimilar action (synonyms: response additivity, Bliss independence) is probabilistic independence. This means that the compounds in the mixture do not interfere with each other but they all contribute to a common result. The model assumes that the compounds in the mixture do not act by the same mode of action and the nature and site of action may also differ among the compounds (Reffstrup et al. 2010).

Interactions are defined as combined actions resulting in a stronger (synergism) or weaker (antagonism) effect than would be expected based on the assumption of additivity. Interactions can be divided into direct chemical-chemical, toxicokinetic and toxicodynamic actions. It is difficult to predict toxic interactions. If the actual exposure is very low it is often unclear whether knowledge about the combined action at higher concentrations is relevant for the low exposure level. Overall, it is generally anticipated that interactions will not appear at relatively low exposure levels since they are primarily caused by various thresholds and saturation phenomenon (saturation of activating, detoxification or reparative processes) (Reffstrup et al. 2010).

Risk assessments of pesticides normally result in the establishment of acceptable daily intakes (ADI) for individual pesticides which are based on so-called points of departure (No Observed Adverse Effect Levels (NOAELs) or benchmark doses) by using a large uncertainty factor. Exposures below these levels are usually considered safe. However, the experimental evidence on mixture effects provokes the question as to whether there is sufficient protection also against combined exposures when each component is present below their individual threshold doses. Experimental studies have produced strong evidence that mixture effects may arise when several chemicals are combined at doses around, or below their points of departure. The majority of these studies have analyzed the effect of combinations composed of chemicals that interact with the same sub-system of an organism. In such cases, the concept of dose addition is applicable. The principles of dose additivity mean that mixture effects are to be expected even when each chemical is present below zero-effect levels, because it is assumed that all toxicants in the mixture behave as if they were a dilution of one another. Hence, any concentration of any compound needs to be considered because it adds to the mixture concentration. This implies that all compounds contribute to the mixture toxicity in direct proportion to their concentration in the mixture and their individual potency. Whether the individual concentrations in the mixture are above or below the corresponding effect thresholds does not matter. This has been demonstrated repeatedly for a broad range of mixtures in toxicological studies (Kortenkamp et al. 2009).

The toxicity of the simultaneous administration of two or more chemical has been investigated in several experiments. In 2008 the PPR Panel carried out a search of the PUB-MED database from 1980 onwards using the search terms “toxicity and mixture”. While there are many papers in the scientific literature concerning the toxic effects of mixtures, both *in vivo* and *in*

vitro, only a few were found that addressed combined effects from dose levels at or below the NOAELs for the compounds when tested individually (EFSA,2008).

The evidence reviewed by EFSA does not exclude the possibility that interactions could in some circumstances lead to toxic effects from combinations of pesticide residues at doses below their individual NOAELs. Only a limited number of compounds have been tested in combination at low doses; and for those combinations that have been tested, interactions might in some cases have been missed because of the spacing of the dose levels that were employed. However, the available evidence support the view that significant toxic interactions between chemicals are much less likely to occur at doses below the effect levels for individual component compounds than at higher doses. To this extent, interaction is less relevant to risk assessment for pesticide residues in food (EFSA, 2008). However, it was recommended that a case-by-case approach should be adopted to consider whether it is biological plausible that interaction might occur between specific pesticides at low, non-effective doses. In some cases, ad hoc tests might then be required (EFSA, 2008).

Concerning dose addition EFSA found that this might occur when certain mixtures of chemicals are administered at relatively low doses. With regard to pesticide residues in food, this form of combined toxicity therefore requires further systematic consideration.

In their 2008 opinion (EFSA, 2008) the PPR Panel proposed to follow a step-wise approach to identify a common assessment group (CAG) consisting of pesticides having a common mode of action. The proposal was mainly based on US EPA documents (EPA, 1999; 2002) and an ILSI report (ILSI, 1999).

The proposed approach included the following steps:

- Preliminary identification of a candidate set of substances that might cause a common toxic effect by a common mode of action. This preliminary grouping is based on one or more of the following criteria:

  Chemical structure. This can be explored by substructure searches in databases for toxophore (a structural feature or moiety contained in substances causing the same toxic effect. The toxic effect is attributed to the interaction of such a feature or moiety with the molecular target) (or a metabolic precursor of a toxophore), core molecular structure, and functional groups;

  Mechanism of pesticidal action. This is considered informative because it is not uncommon that pesticides are toxic to humans through a mechanism that is similar to that of their activity against their target pests;

  General mode/mechanism of mammalian toxicity;

  A particular toxic effect. It is conceivable that similar toxic effects by different compounds might be caused via a common mode/mechanism. This criterion might allow the identification of structurally unrelated substances that act by the same mode of action. It is emphasized that non-specific effects such as body weight changes or death can result from many unrelated factors and consequently are of limited value in identifying potential candidate substances for a common mode/mechanism group (CMG).

- Definitively identify those substances that cause a common toxic effect(s). This step allows a first refinement of the preliminary grouping described above. This is performed by detailed evaluation of available toxicology data for each substance and those not causing a common (i.e. concordant in both site and nature) toxic effect are excluded.
- Determine the toxic mode/mechanism of action by which each substance causes a common toxic effect. While desirable, not all of the specific biochemical events leading to toxicity need to be known or completely characterized. A minimum set of data is required to identify those events that are most crucial in causing the toxicity (mode of action). All available reliable sources of information should be used, including published literature, and textbooks.
- Compare the mechanisms of toxicity/modes of action of the different substances.
- Refine groupings by excluding substances that cause a common toxic effect by a different mechanism/mode of action.

The PPR panel was aware that such a detailed evaluation up to the last step might not be necessary or even possible in all cases. The identification of a CAG is complicated and it is anticipated that it often has to be based on a relative broad set of criteria due to absence of information/data on mechanism/ mode of action. The assessment of the available data therefore requires expert judgement involving knowledge on toxicological effects and their underlying mechanisms. This involves several scientific disciplines, such as biology, biochemistry, molecular biology, and pathology.

An additional consideration arises from evidence in the literature that certain endocrine active compounds in mixture show a dose-additive common effect even if they do not share the same primary molecular target and when exposures are at or below their individual NOAELs (Kortenkamp, 2007, EC 2009, Christiansen et al. 2008, Moretto, 2008, Kortenkamp and Hass 2009, Jacobsen et al, 2010, Reffstrup et al, 2010). Therefore, the issue is the definition of the concept of common mode of action (MOA), and what this would mean for endocrine active compounds. For instance, compounds affecting male sexual development via interference with steroid synthesis and not by antagonism of the androgen receptor would not be grouped according to a narrow definition of MOA whereas it has been shown that a mixture of such compounds results in a dose additive effect (Gray et al., 2001; Hotchkiss et al., 2004). Similar considerations can be applied to estrogenic or estrogen-like chemicals (Picard, 2003). Therefore, it appears that in these cases the criterion for grouping should rather be that of a common phenomenological effect (e.g.: altered ano-genital distance for anti-androgens) (Kortenkamp, 2007). To embrace all scientific issues regarding the appropriate grouping of compounds for cumulative risk assessment, a group of compounds for which a cumulative assessment is thought to be required will be defined as a common assessment group (CAG). Hence, CAGs are not always identical to common mechanism groups (CMG).

Following the general opinion of the PPR Panel (EFSA 2008), the proposed tiered approach to assess cumulative effects from exposure to pesticides was tested in practice for a selected group of triazole pesticides (EFSA 2009). Cranio-facial malformation was selected as the

common endpoint for acute effect of seven compounds and hepatotoxicity was selected as the common endpoint for the chronic assessments of 11 compounds.

The PPR Panel used the following tiers for the hazard characterisation 1) ADI, ARfD; 2) "ADI", "ARfD", adjusted for the common endpoint; 3a) NOAEL for the common endpoint; 3b) BMD for the common endpoint. Several exposure scenarios were considered, including exposure relevant for MRL-setting. The risk assessment was performed for each scenario by calculating the Hazard Index (HI), an adjusted HI, and using the Relative Potency Factor (RPF) method, where the RPF method was applied using either NOAELs or BMDs as Reference Point (RfP).

This example proved to be very valuable in testing the methodology and identifying the necessary next steps before its routine application by EFSA could be recommended.

The PPR Panel concluded that the previously proposed tiered approach could be simplified by starting with a CAG as refined as the data allow and using the same CAG in all steps of the assessment, and restricting each exposure scenario to two tiers, one deterministic and one probabilistic tier.

The Panel concluded that the establishment of relevant CAGs is the starting point for all cumulative risk assessments. Consensus should be reached at an international level on the criteria and compounds that should be used to create a CAG, to avoid differences between national cumulative risk assessments. An important issue is that a first tier should be more conservative compared to the next tiers. In itself, the hazard assessment tiers are clear and could be performed for any CAG.

The present project explores the existing data on pesticide active substances to identify the toxicological effects and endpoints that can be the basis of a cumulative risk assessment. Furthermore proposal for common assessment groups of active pesticide substances having these identified effects and the related endpoints is given with the aim to set the basis for carrying out cumulative risk assessments on a routine basis.

The project thus aims to identify specific adverse, toxic effects considered for cumulative risk assessment (CRA) and as a consequence as far as possible identify effects not relevant in this context. In a searchable database, the outcome of the project will contribute to an overview of toxicological data on pesticide active substances in a manner which facilitate the identification of adverse, toxicological specific effects of the single substance including the dose or NOAEL for these specific effect and where possible data on mechanism or mode of action.

OBJECTIVES

The objectives of the project are:

- To provide EFSA with a clear and concise report that identifies the toxicological effects and endpoints that can form the basis for allocating the active pesticide substances included in Annex I. of Council Directive 91/414/EEC (up to 31st of May 2009) into common assessment groups (CAG) thereby setting the basis for carrying out cumulative risk assessment (CRA) of pesticides on a routine basis in MRL setting.

- To prepare a searchable database of the effects identified as important for cumulative risk assessment listing the pesticide active substances having these effects with the related endpoints together with their no observed adverse effect levels.
- To propose specific common assessment groups of the pesticides for consideration in MRL setting.

Working strategy

1. Working plan

The original working plan suggested by the tenderer was discussed at an interim meeting with the Project Steering Group. Based on the experiences obtained so far in the project it was agreed to change the strategy to make the work more efficient. The new strategy involved the following six steps:

- *Step one*. Identify the pesticide active substances, which are considered relevant for inclusion in CAGs, i.e. exclude the pesticides which are not considered relevant for inclusion in a CAG. This is addressed in Chapter 2.
- *Step two*. Collect information from Annex 1 of Council Directive 91/414/EEC on chemical identity and pesticidal mode of action of each of the included pesticide active substances. The information is included in a working spreadsheet.
- *Step three*. Identify the toxicological targets (effect points) for each pesticide active substance based on the DAR or similar information and add to the working spreadsheet.
- *Step four*. Collect detailed toxicological information for each active substance and add to the working spreadsheet. The data collection should be sufficient to identify possible CAGs. At this stage, the available information on mode or mechanism of action is also recorded. Word documents are prepared for each target organ or tissue with short descriptions of each specific effects and mode/mechanism of action.
- *Step five*. Based on the information and the Word documents obtained at step four, chapters are included in the final report outlining the potential CAGs that can be established for each of the specific toxicological endpoints.
- *Step six*. The information added to the working spreadsheet is imported into a searchable Microsoft Access database.

At the interim meeting it was also agreed to include toxicological active metabolites in the database, where relevant information is reported in the DAR. This is done during the collection of data from the DARs for all target organs and tissues and a complete file containing all these substances will be finalised when all working spreadsheets are finalised.

2. Identification of active substances considered relevant for cumulative risk assessments (CRA)

All the 344 active substances included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009) are listed in Appendix A of this report. However, not all of these substances are suited for inclusion in CRA and therefore, should not be part of a CAG.

The main criteria for further considerations of an active substance for inclusion in a CAG are that the substance is chemically well-defined and that adequate toxicological data are available. The rationales for the exclusion of in total 120 of the 344 active substances included in Annex I of Council Directive 91/414/EEC for further considerations for inclusion in CAGs are presented below:

Twenty-five of the active substances included in Annex I of Council Directive 91/414/EEC are micro-organisms (virus, bacteria, fungi etc.) (42, 45-50, 89, 96, 173, 195, 214, 235, 236, 247, 269, 278, 300, 302, 326-330, and 339). As these active substances are not chemicals, they are irrelevant for inclusion in CAGs. These substances appear on an orange background in Appendix A of this report.

Straight Chain Lepidoptera Pheromones (SCLPs) is a group of pheromones naturally produced by insects in the order Lepidoptera, which includes butterflies and moths. The SCLPs are characterised by an unbranched aliphatic chain (between 9 and 18 carbons) ending in an alcohol, aldehyde or acetate functional group and containing up to 3 double bonds in the aliphatic backbone and thus, not chemically well-defined. Twenty-seven active substances belonging to the SCLPs group are included in Annex I of Council Directive 91/414/EEC (1-24, 124, 301, and 311). Furthermore, it is concluded in the SANCO Review Report (SANCO/2633/08) "... there are clear indications that it may be expected that Straight Chain Lepidopteran Pheromones, when they are applied via retrievable sized dispensers, do not have any harmful effects on human or animal health ...". In addition, no ADIs have been established for the SCLPs (stated as 'not applicable' in Annex I of Council Directive 91/414/EEC). For these reasons, the SCLPs are considered as irrelevant for inclusion in CAGs. These substances appear on a dark green background in Appendix A of this report.

For fifteen of the active substances included in Annex I of Council Directive 91/414/EEC, there is a cross reference to another active substance in the remark column. As these substances were grouped due to similar chemical and toxicological properties, only one substance in the group has to be considered for inclusion in CAGs. For Bordeaux mixture (62), copper hydroxide (91), copper oxychloride (92), cuprous oxide (93) and tribasic copper sulphate (324), there is a reference to "copper compounds" (90) and all the substances are covered by the toxicological data for copper compounds and will be placed in the same CAGs as copper compounds. For capric acid (68), caprylic acid (69), fatty acids C7-C18 and C18 unsaturated potassium salts (139), fatty acids C8-C10 methyl esters (140), lauric acid (194), methyl decanoate (220), methyl octanoate (222), and pelargonic acid (241), there is a reference to "fatty acids C7 to C20" (138) and all the substances are covered by the toxicological data for fatty acids C7-C20 and will be placed in the same CAGs as fatty acids C7-C20. For quizalofop-P-ethyl (283) and quizalofop-P-tefuryl (284), there is a reference to "quizalofop-P" (282) and these substances are covered by the toxicological data for quizalofop-P and will be placed in the same CAGs as quizalofop-P. It should be noted, however, that the toxicological information for quizalofop-P in fact is for quizalofop-P-ethyl

and quizalofop-P-tefuryl. The fifteen cross referenced substances appear on a blue background in Appendix A of this report.

Twenty-four of the remaining 277 active substances included in Annex I of Council Directive 91/414/EEC are either complex mixtures or in other ways not a chemically well-defined substance (61, 135, 137, 138, 170, 177, 191, 193, 197, 237-240, 244, 254-257, 285-288, , and 294). These substances, which appear on a red background in Appendix A of this report, are irrelevant for inclusion in CAGs.

For 22 of the remaining 253 active substances included in Annex I of Council Directive 91/414/EEC (25, 32, 38, 41, 66, 72, 106, 109, 111, 112, 132, 171, 176, 221, 230, 258, 270, 279, 305, 334, 338, and 340), an ADI has not been established (stated as 'not applicable' in Annex I of Council Directive 91/414/EEC). For the major part of these substances (25, 32, 38, 41, 66, 72, 106, 132, 171, 221, 230, 258, 270, 279, 334, and 338), it is concluded in the respective SANCO Review Reports "... there are clear indications that it may be expected that XXX does not have any harmful effects on human or animal health ...". For three of these substances (109, 111, 112), it is concluded in the respective SANCO Review Reports "With particular regard to residues, the review has established that the residues arising from the proposed use will not be significant and … they will have no harmful effects on human or animal health.". For one substance (176), it is concluded in the SANCO Review Report "Based on the proven low toxicity of the active substance from the available toxicity studies, it was not necessary to set any of the following reference values: ADI, ARfD or AOEL.". For one substance (305), it is concluded in the SANCO Review Report "... for an essential element as sulphur, exposure is evaluated against background levels and sulphur is considered to be low risk.". For one substance (340), it is concluded in the SANCO Review Report "Since the plant protection products containing warfarin are not used on plants or plant products, use, consequent on application consistent with good plant protection practice, has no harmful effects on human or animal health.". For two of the remaining 231 active substances included in Annex I of Council Directive 91/414/EEC (36, 65), data are insufficient to consider the inclusion of the substances in any CAG (only acute toxicity data have been submitted). For these two substances (36, 65), it is concluded in the respective SANCO Review Reports "... there are clear indications that it may be expected that XXX does not have any harmful effects on human or animal health ...". Two of the remaining 229 active substances included in Annex I of Council Directive 91/414/EEC are inorganic substances (148, 188). For one of these substances (148), it is concluded in the SANCO Review Report "... no harmful effects on human or animal health will be arising from the proposed uses ...". For the other substance (188), it is concluded in the SANCO Review Report "... there are clear indications that it may be expected that iron sulphate does not have any harmful effects on human or animal health ...". As all these 26 active substances are considered as having no harmful effects on human health (SANCO Review Reports), these substances are considered irrelevant for inclusion in CAGs. These substances appear on a yellow background in Appendix A of this report.

For 3 of the remaining 227 active substances included in Annex I of Council Directive 91/414/EEC (160, 231, 275), the DAR was not available neither from the CIRCA database nor from the Danish Environmental Protection Agency. The Rapporteur Member States (RMS) have been contacted by email twice, but the DARs are still missing. Therefore, these

substances, which appear on a pink background in Appendix A of this report, have also been excluded for further considerations for inclusion in CAGs.

In total, 120 of the 344 active substances included in Annex I of Council Directive 91/414/EEC have been excluded for further considerations for inclusion in CAGs. The remaining 224 active substances to be evaluated for inclusion in CAGs appear on a light green background in Appendix A of this report and are also listed in Appendix B of this report.

3. Collection of information

3.1. Chemical structure and pesticidal mode of action

The specific numbers for the pesticide active substances used in the present project refers to the numbers according to an alphabetic ordering of the compounds (Appendix A and Appendix B of this report).

Information on the chemical identity and pesticidal mode of action of the active substances was obtained from the Pesticide Properties Database (PPDB 2009) and the Pesticide Manual (2010). The following information on the chemical identity as well as the mode of action of each substance was included in a working spreadsheet: Substance name according to the CIRCA database (with possible alternative names also included), pesticide category, chemical group, IUPAC name, CAS Register No, and EC No if available. The chemical structure of each substance was drawn based on the SMILES code (available in the PPDB) by using the ChemDraw program. The structures were saved as the ChemDraw filetype '.emf' (this can be imported to Access databases) and included in a separate document, as agreed with the database developer.

The information that substances contain similar chemical structures or have the same pesticidal mode of action may indicate a possible similar toxicity to experimental animals and humans. In this project this information is a part of the quality control. When a CAG is identified it will be checked if there are pesticide active substances with similar chemical structure or the same pesticidal mode of action that were not included. If that is the case, it will be checked if it was a mistake not to include this/these substances in the CAG.

3.2. Toxicological effects and mode/mechanism of action

Information on toxicological effects and potential modes and/or mechanisms of action for mammalian toxicity were obtain from Draft Assessment Reports (DARs), DAR addenda, and EFSA/ECCO/EPCO peer review reports. Reports from the Joint FAO/WHO Meting on Pesticide Residues (JMPR) were consulted in order to examine whether additional relevant toxicological information was available. In addition, a search in the open literature, such as PubMed, was performed on additional toxicological studies and potential modes/mechanisms of action of the active substances.

Initially, DARs for the included active substances were examined for toxic effects on specific organs or tissues. On the basis of this examination, Excel spread sheets were established for each individual organ/tissue system containing the following information: Substance number, substance name, phenomenological/specific toxicological effect(s), study type (species, strain,

duration, mode of administration), mode of action, mechanism of action, NOAEL, LOAEL, reference/source, and remarks.

Due to the organisation of the work load where all the studies were to be scrutinised further by experts in the specific toxicological areas it was decided to group some traditional endpoints into a larger common group. An example was that effects on fertility and fetal development were grouped under the heading reproductive and developmental toxicity. The term effect point was therefore used for either a traditional endpoint (toxicological target) or a group of traditional endpoints.

For all the effects identified in the DARs the relevant studies were evaluated by the experts allocated to the various effect areas. As the purpose of this project was not to review the DARs the information therein was considered valid. Consequently, the evaluations regarding a particular effect of an active substance to be treatment-related or not, as well as the NOAELs and LOAELs for the effects observed in the individual toxicological studies were taken from the DARs whenever available.

For many active substances, the effect observed for a particular target organ/tissue the changes were small in comparison to the control group, did not reach statistically significance, were not dose-related, reported in only one or a few studies, and/or findings were not consistent across studies, sex and/or species. Therefore, the findings were often considered in the DARs not to be treatment-related. For findings in long-term studies, the findings were often considered in the DARs to be age-related – not treatment-related. Thus, a particular target organ/tissue in such cases seems not to be a primary target organ/tissue for the active substances included in this project and the substances identified to affect a target organ/tissue in such cases were not considered further for CAGs in this report. Moreover, the description of the findings in the various DARs are very different regarding details and exactness, which contributes to uncertainties in the interpretation of the findings described in the DARs.

In many cases, the NOAELs and LOAELs for the specific effects were not identical with the study NOAELs and LOAELs. In these cases, the NOAELs and LOAELs were carefully evaluated by the experts based on the information in the DARs and relevant comments are presented in the remark column of the spreadsheets. The NOAELs and LOAELs in the spreadsheets are generally expressed as a systemic dose in the unit ‘mg/kg bodyweight/day’ regardless of the exposure route / mode of administration. For dietary studies (and inhalation studies) where the amount of the test substance is expressed as a concentration in the feed (or in the test atmosphere), the systemic dose was cited from the DAR whenever available. In other cases the systemic dose was calculated from the dietary concentration by using the general conversion factors provided in the OECD ‘Guidance notes for analysis and evaluation of chronic toxicity and carcinogenicity studies’ (OECD 2002) and presented in Appendix C of this report.

However, more recent information from DAR addenda, and EFSA/ECCO/EPCO peer review reports was obtained, when available, and JMPR reports were also consulted for additional information. The open literature was searched via PubMed. Pubmed was mainly used for the literature searches as this database is the most comprehensive one covering toxicological topics of relevance for human health, including information of modes/mechanisms of action. For all active substances identified to affect a target organ/ tissue, the search was performed for all the substances by name and by CAS number. If the number of hits were below 100, all

abstracts were checked. If more than 100 hits, the search was refined to include the name of the specific target organ/tissue. The literature search for active substances on reproductive and developmental toxicity was slightly different from the general search strategy and is described in section 25.2.2. Unless otherwise stated, the introductory text for a specific target organ/tissue is based on relevant text books (without a reference to these text books) and the information on specific substances is taken from the respective DAR (without a reference to the DARs either located from the Circa database or from the rapporteur on request if not available from the Circa database).

The quality control was performed in two steps:

- *The first step* consisted of a multilateral quality check in form of an in-house meeting with participation of all the experts involved in this project. The individual effect point sections included in the preliminary draft report for discussion at the fourth Steering Group meeting held on 15-16 March 2011 in Parma were presented and discussed at the meeting. The in-house meeting was considered as being more feasible and valuable for the project instead of a consultation with external toxicologists as the expertise on the various toxicological targets and effect points is represented by the in-house experts. This decision was agreed at the third Steering Group meeting held on 13 October 2010 (teleconference).
- *The second step* consisted of a quality check of both the individual effect point spreadsheets and sections for the report. This step was predominantly performed by the senior experts involved in the project.

Based on the evaluations of the relevant studies and the quality control final versions of the individual effect point spreadsheets and sections for this report were prepared.

The report sections for the various target organs/tissues are kept as consistent as possible, i.e. following a predefined template. However, as target organs/tissues are different, the report sections will consequently be somewhat inconsistent reflecting the differences between the target organs/tissues.

4. Criteria for dismissing effects for CAG

Clearly, effects reported in toxicological studies that are considered to be either not adverse or of no relevance for human risk assessment should not be used for establishing CAGs. It is outside the scope of this report to prepare a comprehensive list of non-adverse effects and effects without relevance for humans. However, at number of examples relevant for the pesticide active substances can be found in the chapters dealing with CAGs for the different toxicological target organs and tissues. Examples are non-adverse discoloration of an organ/tissue, for instance the bone marrow, and development of thyroid tumours in rats and mice because of changes in the thyroid and pituitary hormone levels, considered to be of no relevance for humans.

However, there are a number of effects that can be considered adverse and also of relevance for humans but nevertheless are not considered relevant for inclusion in a CAG. These non-specific or indirect effects can occur either as a result of an advanced state of a toxic

impact/insult i.e. a secondary effect to a specific (direct) effect or as a consequence of high, massive (unrealistic) exposure to a pesticide active substance.

Acute effects like mortality and clinical signs of toxicity will not be taken into consideration as CRA is generally only relevant following cumulative exposure to the active substances over time.

Clinical observations will constitute a major part of the group of non-specific effects as they often occur as a consequence of a substantial toxicological impact or an advanced state of toxicological injury leading to a terminal effect including "near death" . These effects represent the state beyond which the resources of the organism are exhausted in compensating for an effect of a toxicological insult. Clinical observations/effects reported from acute toxicity tests including the LD_{50} tests are often of such nature e.g. decreased activity/not alert, subdued appearance, persistent recumbency, catalepsia, lethargy, comatose, sedation, lacrimation, chromodacryorrhea, salivation, nasal discharge, diarrhoea, pinched abdomen, hunched posture, distended abdomen, piloerection, laboured respiration, pinched abdomen, ataxia, paresis, tremor, convulsion etc. Many of these clinical observations especially the latter four mentioned could very well be referred to a neurotoxicological MOA. However, this is not done unless data are available that allow for categorizing these effects as a result of a specific MOA contrary to a terminal state of an un-specific toxicological effect.

Effects on body weight and related parameters such as decreased body weight (BW), decreased body weight gain (BWG), decreased food consumption (FC), decreased water consumption, dehydration, soft stool, etc. seen after repeated exposure are also examples of unspecific effects as a consequence of exposure to a toxicant or simply as a secondary effect of a specific toxicological effect where the organisms resources to adapt are depleted or exhausted. Where effects like decrease in BWG or FC are the only effects observed even at low doses they will also be referred to the category of non-specific effects due to lack of data to establish a specific MOA.

Changes in organ weights are generally only considered relevant if the change is relative, i.e. relative to the bodyweight or brain weight:

- When the changes in the absolute weight of an organ is recorded together with similar changes in absolute weights of other organs and these changes relate usually to changes in body weight and are not accompanied with any pathological changes the change is considered as an unspecific effect, which is not related to toxicity to the organ.
- When the changes in the relative weight of an organ (alone or accompanied by a change in absolute organ weight) alone or accompanied by changes in relative (and absolute) weights of other organs without pathological changes and which can be related to changes in body weight (i.e. secondary to body weight changes) this is considered an indirect effect, which is not related to toxicity per se although it is a treatment related effect.
- When the changes in the relative weight of an organ (alone or accompanied by a change in absolute organ weight) not necessarily accompanied by changes in relative (and absolute) weights of other organs, but accompanied by dose related morphological changes in the organ this is considered to be a direct effect.

Many active substances were reported to increase or decrease the relative weight of a target organ or tissue. In general, the changes were small in comparison to the control group, did not reach statistically significance, were not dose-related, increased weights were related to an increased incidence of masses (noted in the macroscopic examination) and/or neoplasms (noted in the microscopic examination), observed in only one or a few studies, and/or findings were not consistent across studies, sex and/or species. Therefore, the changes in relative organ weight were often considered in the DARs not to be treatment-related. Moreover, the description of the findings in the various DARs are very different regarding details and exactness, which contributes to uncertainties in the interpretation of the findings described in the DARs. In conclusion, increased and decreased relative organ weight are considered as not being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for effects on a specific target organ or system.

Changes in white blood cell parameters are not recommended for CRA, see section 14.2.2.4.

Changes in blood and urine clinical biochemistry parameters, such as hepatic leakage enzymes (i.e. alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), ornithine carbamyltransferase (OCT), and sorbitol dehydrogenase (SDH)) and creatinine are indirect indicators for damage to various organs, in particular the liver and kidneys. However, the CAGs for different morphological/histopathological effects on the tissues will cover more adequately the potential injury indicated by these blood and urine parameters.

Some effects like *increased plasma cholesterol* are usually considered non adverse if the increase is under a certain limit. However, in cumulative risk assessment it is not relevant to distinguish between adverse/not adverse effects based on such limits because the accumulated increase caused by several substances each contributing with an increase under the specified limit may exceed the limits. Effects which are considered non adverse under a certain limit in traditional risk assessments may therefore be considered adverse in cumulative risk assessment. As a consequence, no effects will be considered non adverse based on a limit of the effect.

Experimental animals or humans may adapt to chemicals. In toxicological experiments an effect is considered adaptive if the effect is observed after relative short time of exposure but vanish after prolonged exposure. There are some discussions whether this should be considered adverse. Although there are no toxic effects after long-time exposure to such substances some scientist consider adaptive effects adverse because the organism clearly uses some of its “spare capacity” to deal with this single substance. As it cannot be excluded that an organism will not be able to adapt to several substances simultaneously an effect is considered adverse even though the effect vanish after prolonged exposure.

The above text represents general considerations of effects which are referred to as non-specific (indirect) in the present report. However, many more examples are given under the specific target organs/tissue and moreover those effects not considered/listed as the basis for establishing the CAG can be taken as non-specific effects according to the consideration above.

5. Considerations on mode / mechanism of action

The initial step in the cumulative risk assessment of a mixture of chemicals is to identify one or more groups of compounds that induce a common toxic effect by a common mechanism of toxicity. Such compounds belong to a "common mechanism group" (CMG) for which dose-addition (simple similar action) apply. When chemicals in a mixture act in the same way, by the same mechanism or mode of action, and differ only in their potencies dose-addition implies that the effect of exposure to the mixture is equivalent to the effect of the sum of the potency-corrected doses of each component.

The International Life Sciences Institute (ILSI) has considered the definition of the term common mechanism of toxicity. It was concluded that chemicals act via a common mechanism of toxicity if they cause the same critical effect, act on the same molecular target tissue, act by the same biochemical mechanism of action, or share a common toxic intermediate (ILSA 1999).

The toxicological literature on mixtures and regulatory guidance documents for mixture assessment often fail to make clear distinctions between the terms **mode of action** and **mechanism of action**.

In 1999, the US EPA provided "Guidance for identifying pesticide chemicals and other substances that have a common mechanism of toxicity" (EPA 1999a). In this guidance US EPA defined a common mechanism of toxicity to be caused "by the same, or essentially the same, sequence of major biochemical events." This definition is equivalent to the definition of the term mode of action used by US EPA in 2002 (EPA 2002). In other reports US EPA distinguished between mechanism of action and mode of action: The term mode of action describes "the key events and processes starting with interaction of a compound with a cell via operational and anatomical changes, resulting in the toxic effect. Mechanism of action implies a more detailed understanding and description of the steps at the molecular level" (EPA 2000, 2005).

EFSAs PPR Panel also noted that in some US EPA documents on cumulative risk assessment, when the term mechanism of action is used it implies mode of action as defined above. Thus, a common mechanism of toxicity was defined as occurring when "two or more pesticide chemicals cause a common toxic effect by the same, or essentially the same, sequence of major (or key) biochemical events" (EFSA 2008).

According to Borgert et al. (2004) mechanism of action denotes the molecular sequence of events leading from the absorption of an effective dose of a chemical to the production of a specific biological response in the target organ. Mode of action is a more general description of the chemical action. It refers to the type of response produced in an exposed organism or to only critical steps or features of the mechanism required for the production of the particular biological response. Therefore, when determining similarity between chemicals in a mixture, chemicals may not appear adequately similar to support use of an addition model when looking for a similar mechanism of action, but may appear adequately similar when looking for a similar mode of action. Thus, the mode of action is known if the full mechanism is known, but the reverse is not true. (Borgert et al. 2004).

Lambert and Lipscomb (2007) considered that "*mode and mechanism of toxic action are* ***mutually supportive concepts*** *that differ only in the level of detail regarding the processes*

involved in toxicity". They cited the definitions originally given by the US EPA (EPA 2000) and stated that mechanism of action typically has been relegated to molecularly discrete reactions (e.g. DNA/RNA modifications, enzyme-substrate interactions, oxidative stress), while mode of action has commonly been described at a more macrospopic level of biological organisation. For example a toxic mode of action has been decribed as a set of physiological and behavioural sign characteristic of an adverse biological response.

Teuschler (2007) discussed the importance of having a clear set of definitions and ideas of the concept of similarity of toxic action in order to develop a common understanding among risk assessors. She pointed out that "*similarity of toxicological action represents a **continuum** of information ranging from a high level of detail regarding the molecular basis of the toxic effect (mechanism of action) to knowledge of key cellular and biochemical events (mode of action), to a low level of knowledge regarding a general toxicological effect at the target organ level*" (toxicological similarity) (Table 5.1).

Table 5.1. The continuum of information behind similar toxic action (after Teuschler 2007).

Terminology describing toxicological action	**Mechanism of action**	**Mode of action**	**Toxicological similarity**
Level of knowledge needed	High	Medium-High	Low
Likelihood of having knowledge	Low	Low-Medium	High
Interpretation of terminology	Knowledge of the details of the molecular basis of the toxic effect	Knowledge of the sequence of key cellular and biochemical events (measurable parameters) that result in a toxic effect	General knowledge of toxicological effect at the target organ level

According to the International Programme on Chemical Safety (IPCS 2009b) mode of action (MOA) is "*a biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data. A mode of action describes key cytological and biochemical events - that is, those that are both measurable and necessary to the observed effect- in a logical framework*" (IPCS 2009b). The mode of action contrasts with mechanism of action, which generally involves a sufficient understanding of the molecular basis for an effect and its detailed description, so causation can be described in molecular terms (Boobis et al. 2006). IPCS (2009b) has defined mechanism of action as "*the*

specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system".

The IPCS originally developed a framework for establishing the mode of action of chemical carcinogens in animals. The first step is to determine whether the weight of evidence based on experimental observations is sufficient to establish a hypothesized mode of action. This comprises a series of key events causally related to the toxic effect, identified using an approach based on the Bradford Hill criteria for causality (Sonnich-Mullin et al. 2001). This mode of action framework was later updated and incorporated into the IPCS framework for analysing the relevance of a cancer mode of action for humans (Boobis et al. 2006). In 2008 the IPCS mode of action framework was extended to embrace also non-cancer endpoints and was incorporated in the IPCS framework for analysing the relevance of a non-cancer mode of action for humans (Boobis et al. 2008).

The present report agrees with Teuschler (2007) in that mode of action and mechanism of action represents a continuum of information that includes different sets of mechanistic information. For the purpose of this report mechanism of action (common assessment group (CAG) level 4) denotes detailed, stepwise information at various levels of biological organization whereas mode of action (common assessment group (CAG) level 3) includes only the critical mechanistic steps that produce a characteristic biological effect.

As already mentioned in the introduction, evidence in the literature suggest that certain endocrine active compounds having a common phenomenological effect in mixture show a dose-additive common effect (such as for instance altered ano-genital distance for anti-androgenic compounds) when exposures are at or below their individual NOAELs even if they do not share the same primary molecular target. Therefore, it appears that the first criterion for grouping compounds in a CAG should be that of a common phenomenological effect (toxicological similarity) (common assessment group (CAG) level 2). Hence, CAGs are not always identical to common mechanism groups (CMG).

6. Development of CAGs

The toxicological targets identified in step three for each of the 224 active substances to be evaluated for inclusion in CAGs were (in alphabetical order): Adrenal gland, bone marrow, bones / skeleton, brain, cardiovascular system (including the heart), eye, gall bladder, gastrointestinal tract, haematological system, immune system, kidney, liver, lung, lymph node, mamma, muscle, nervous system, pancreas, parathyroid, pituitary gland, reproductive and developmental toxicity (including reproductive organs), salivary gland, skin, spleen, stomach, thymus, thyroid, and urinary bladder.

For several of the active substances listed in Appendix B of this report, neoplasms observed in the toxicological target organs/tissues were also initially registered in step three. Based on information in the DARs, neoplasms in the target organs/tissue were evaluated for the carcinogenic effect(s) concurrently with the assessments of non-carcinogenic effects for the respective target organs/tissues. Generally, the incidences of tumours at the highest dose levels tested did not reach statistically significance, were within the historical control range and/or were not dose-related. Therefore, the neoplasms were often considered in the DARs not to be treatment-related and consequently, the substances were concluded not to be carcinogenic. It is presumed that a substance is not approved as a pesticide active substance

and included in Annex I of Council Directive 91/414/EEC if the substance is carcinogenic and a genotoxic mode of action has been proposed for the carcinogenic effect(s). Therefore, for the purpose of the CAG project, a non-genotoxic mode of action has been assumed for the substances for which tumours were noted as an endpoint. Consequently, the NOAEL set for the effect(s) leading to the formation of such neoplasms are generally much lower than the NOAEL for the induction of neoplasms and thus, CRA based on a NOAEL set for the effect(s) leading to the formation of such neoplasms will also protect against induction of neoplasms. In addition, certain neoplasms are not relevant for humans, i.e. the induction of neoplasms are generally via a mode / mechanisms of action that is specific for a particular experimental animal. Examples of that include liver tumours in rodents via peroxisome proliferation, renal tumours in male rats via induction of alpha-2-microglobulin, thyroid tumours in rodents via increased serum TSH leading to stimulation of thyroid follicular cell growth and toxicity. In conclusion, the CAG level 2 for neoplasms is generally not recommended for CRA. However, information regarding neoplasms has been included and addressed in several of the endpoint sections when considered of relevance.

At the interim meeting with the EFSA Project Steering Group it was decided that there should be several possible levels for CAGs for a given toxicological target. In the EFSA opinion on triazoles (EFSA 2009) two approaches were considered. An approach consisting in only including substances in a CAG when it has been demonstrated that they actually possess the same mode/mechanism of action; and an approach consisting in including all compounds that show a common phenomenological effect on the target in question.

This led to the suggestion of a tiered approach in which four levels of CAGs would be possible reflecting increased knowledge on the mode/mechanism of action behind the toxicological effect observed. The four levels are 1) toxicological target, 2) common phenomenological effect, 3) common mode of action, and 4) common mechanism of action (see Figure 6.1).

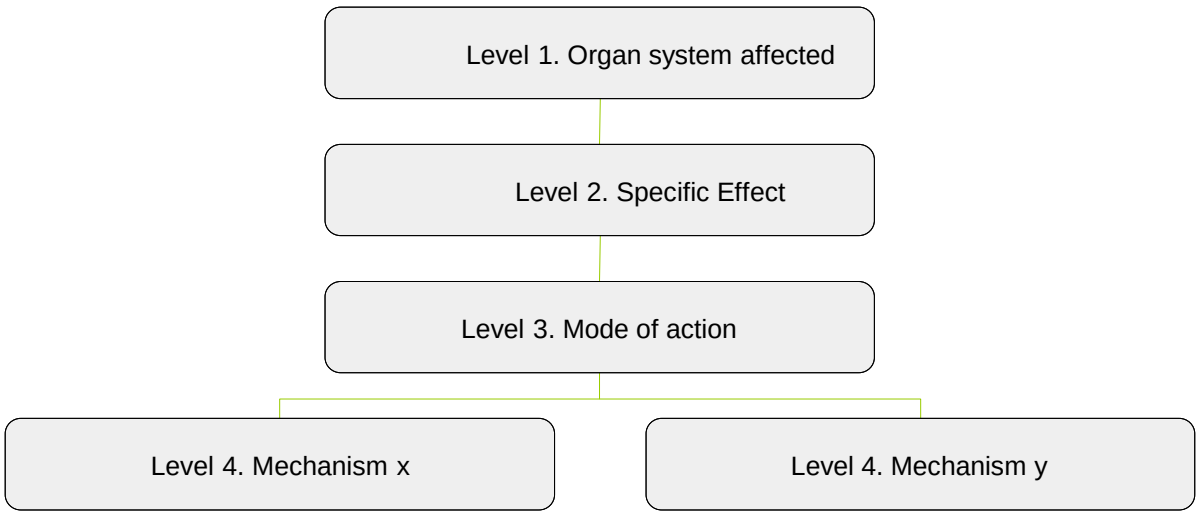


Figure 6.1. Tiered approach for allocating CAGs at different levels

CAG level 1:

All the active substances mentioned in the DARs to have toxicological effects on the target organ/tissue in question were generally allocated to the CAG level 1. This CAG merely collects all the substances that were to be further examined for specific phenomenological effects on the organ/tissue and, if possible the underlying mode/mechanism of action. The CAGs at level 1 should not be used for cumulative risk assessment.

CAG level 2:

A CAG at level 2 includes the active substances that exert a specific phenomenological effect on the target in question. Several CAGs at level 2 may be relevant for each target when different distinct specific effects are induced by different substances. As an example, the active substances that show effect on the thyroid gland were allocated into six distinct CAGs at level 2: Changes in serum T3 (and T4), increased TSH, follicular cell hyperplasia, follicular cell tumours, parafollicular cell hyperplasia, and parafollicular cell neoplasms. As a special case, due to the complexity, the active substances showing reproductive and developmental toxicity were initially allocated to eight distinct CAGs at level 2, which were further subdivided based on a number of specific phenomenological effects.

The CAGs at level 2 are established on the basis of specific phenomenological effects without any prior knowledge about mode/mechanism of action and do therefore not fulfil the criteria for a cumulative mechanism group that can be expected to exert dose additivity. However, risk managers may wish to use a CAG at level 2 in order to consider whether a more realistic cumulative exposure assessment (for instance response addition) to certain active substances would need to be further investigated.

For such an assessment, it can be recommended to use the Reference point index (RfPI) (based on the NOAELs for the compounds in the CAG) as described by the PPR Panel (EFSA 2008). The RfPI is similar to the Point of departure index (PODI) advocated by EFSA (2007) and by WHO (2009) to be used in cumulative risk assessment.

The Reference Point Index (RfPI) represents the sum of the exposures to each pesticide expressed as a fraction of their respective RfPs for the relevant effect (e.g., the dose that causes a 10% effect, BMD10; or the NOAEL). When the RfPI multiplied by a chosen group uncertainty factor (UF) is lower than 1, the combined risk is considered acceptable. An UF of 100 is recommended.

CAG level 3:

For a number of pesticide active substances, information is available that show or permit a hypothesis on the mode of action for several specific effects (reported at CAG level 2), mostly for substances that affect the liver and the endocrine systems (reproductive organs, thyroid) as

well as the nervous system. An example is that a number of active substances which affect the performance of the male reproductive organ system may do so via an anti-androgenic mode of action. The CAGs at level 3 established based on these criteria are considered valid for cumulative risk assessment.

CAG level 4:

For some of the pesticide active substances, studies are available (mainly in the open literature) that point upon a specific mechanism of action for the toxicological effects seen, most notably for endocrine active substances. Most of these studies are *in vitro* studies and care should therefore be taken in the interpretation of the results. However, for many of the active substances the mechanistic effects observed *in vitro* are substantiated by the effects seen in the animal studies and can therefore form the bases for allocation of a CAG at level 4. An example would be that an anti-androgenic mode of action for effect of an active substance on male reproductive performance (reported at CAG level 3) was likely due to an antagonistic effect on the androgen receptor as shown *in vitro*. The CAGs at level 4 are considered valid for cumulative risk assessment.

CAG nomenclature:

The ideal is that an active substance allocated to a specific CAG level 4 could be tracked back to the associated CAG at level 3 and again tracked back to the associated CAG at level 2. Therefore, the following nomenclature has been applied for the CAGs at level 2, level 3 and level 4 for the target organs (excluding reproductive and developmental toxicity) as far as possible.

A CAG at level 2 includes the active substances that exert a specific phenomenological effect on the target in question. Several CAGs at level 2 may be relevant for each target when different distinct specific effects are induced by different substances. A CAG at level 2 is therefore described by a number followed by a letter: CAG level 2a, CAG level 2b etc.

A CAG at level 3 includes the active substances that exert a specific phenomenological effect on the target in question via a specific mode of action. Several CAGs at level 3 may be relevant for each target when different distinct specific effects (CAG level 2) are induced by different mode of actions for the respective substances (CAG level 3). A CAG at level 3 is therefore described by a number followed by a letter and a number: CAG level 3a1, CAG level 3a2, CAG level 3b1 etc.

If a CAG at level 3 is associated with a specific CAG level 2 then:

- A substance allocated to CAG level 3a1 is also allocated to CAG level 2a
- A substance allocated to CAG level 3a2 is also allocated to CAG level 2a
- A substance allocated to CAG level 3b1 is also allocated to CAG level 2b etc.

A CAG at level 4 includes the active substances that exert a specific phenomenological effect on the target in question via a specific mode of action and a specific mechanism of action.

Several CAGs at level 4 may be relevant for each target when different distinct specific effects (CAG level 2) are induced by different mode of actions for the respective substances (CAG level 3) and by different mechanisms of action (CAG level 4). A CAG at level 4 is therefore described by a number followed by a letter, a number and a letter: CAG level 3a1a, CAG level 3a1b, CAG level 3a2a, CAG level 3a2b, CAG level 3b1a, CAG level 3b1b etc.

If a CAG level 4 is associated with a specific CAG level 3 which again is associated with a specific CAG level 2 then:

- A substance allocated to CAG level 3a1a is also allocated to CAG level 3a1 and CAG level 2a
- A substance allocated to CAG level 3a1b is also allocated to CAG level 3a1 and CAG level 2a
- A substance allocated to CAG level 3a2a is also allocated to CAG level 3a2 and CAG level 2a
- A substance allocated to CAG level 3a2b is also allocated to CAG level 3a2 and CAG level 2a
- A substance allocated to CAG level 3b1a is also allocated to CAG level 3b1 and CAG level 2b
- A substance allocated to CAG level 3b1b is also allocated to CAG level 3b1 and CAG level 2b etc.

It should be noted, however, that for some substances for which a mode and/or mechanism of action has been identified, there is no association to a particular CAG at level 2 or 3, respectively. In such cases, the first letter assigned to a particular CAG at level 3 is described by the next letter in the alphabet which is not associated with a CAG level 2 or 3 for a particular target organ. As an example, specific phenomenological effects on the target in question have been allocated to CAG level 2a, 2b, 2c and 2d. If a mode of action (CAG level 3) could not be associated with one of the CAGs at level two, then this mode of action would be assigned to CAG level 3e1. If a mechanism of action (CAG level 4) could not be associated with neither any of the CAGs at level 2 nor the CAG at level 3, then this mechanism of action would be assigned to CAG level 4f1a.

It should also be noted that the general CAG nomenclature has not been applied for the liver as this approach turned out to be too complicated due to the fact that the mode of actions (CAG level 3) in general could be tracked back to a single specific phenomenological effect (CAG level 2). Therefore, a CAG at level 3 is described by a number followed by a letter, e.g. 3a and the associated CAG at level 4 is described by a number followed by a letter and a number, e.g. 4a1, 4a2 etc.

For the thyroid, a slightly different approach has been used as a combined mod of action model has been proposed for CAG level 3. The CAGs at level 4 are therefore, described by a number followed by a letter, e.g. 4a, 4b etc.

General remark:

For some CAGs, there is only one active substance included. However, such potential CAGs are included in the report because novel research may identify such effects for other active substances. In addition, regulatory bodies may in the future consider active substances not included in the present project, i.e. substances adopted for inclusion in Annex 1 of Council Directive 91/414/EEC after the 31st of May 2009.

Consideration of pesticide active substances having similar structures or belonging to the same chemical class:

The PPR Panel (EFSA 2008) suggested that pesticide active substances could also potentially be grouped in a CAG based on similar chemical structure. This could be explored by substructure searches in databases for toxophores (a structural feature or moiety contained in substances causing the same toxic effect. The toxic effect is attributed to the interaction of such a feature or moiety with the molecular target) (or a metabolic precursor of a toxophore), core molecular structures, and functional groups. A detailed substructure search was judged to be outside the scope of this report. However, the CAGs at level 2, 3, and 4 that were established based on the toxicological effects were examined for completeness or not of active substances having similar structures based on the chemical grouping outlined in Appendix A.

Active substances belonging to the same chemical class may have similar toxicological effects. For selected target organs, information in the DARs on effects on the respective target organ has been summarised for evaluation of similarity of toxicological effects within the relevant chemical classes, i.e. the chemical classes containing more than one active substance. The selected target organs include the liver (see section 17.4), the nervous system (see section 21.4) and the eye (see section 11.4).

Results

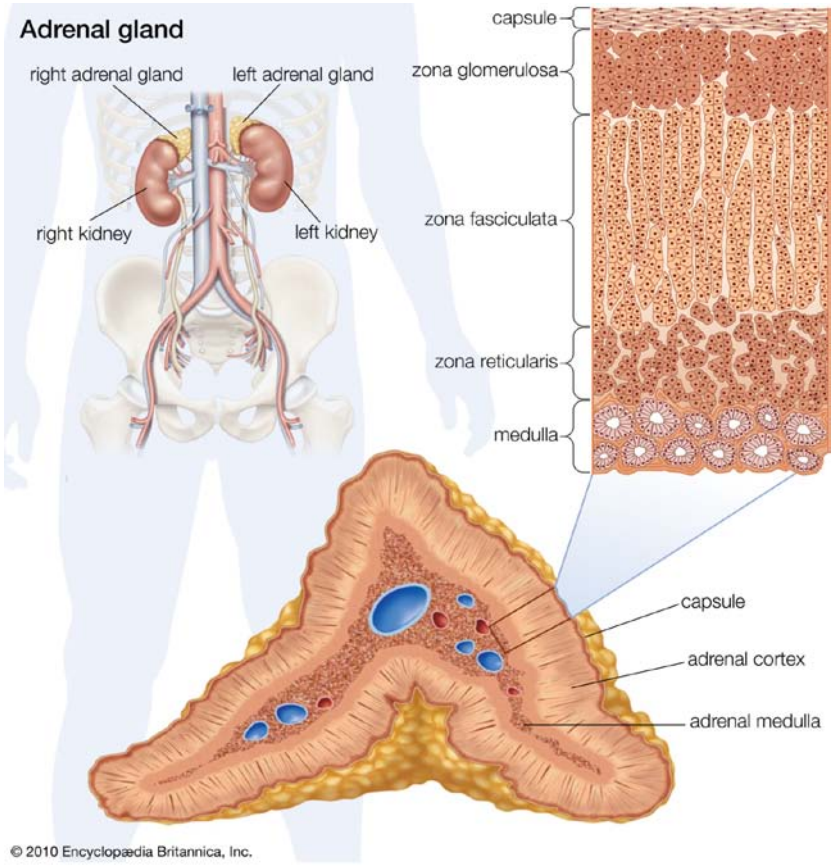
The available toxicological studies on the active substances listed in Appendix B were carefully scrutinized for toxicological effects and CAGs are proposed for a number of specific target organs and tissues. For each target recommendations are given on potential CAGs that should be considered for use in cumulative risk assessment (CRA). The results are describe below for each target organ/tissue (see chapter 7-31).

A searchable database in Microsoft Access has been provided to EFSA. Together with key information on the chemical and pesticidal properties and the acceptable daily intake (ADI) established by the EU for the active substances, the database contains the results of the present evaluations of all the available toxicological studies used to allocate the active substances into different CAGs, including the NOAELs and LOAELs (see chapter 32).

7. Adrenal gland

7.1. Introduction

There are two adrenal glands, one on top of each kidney, see Figure 7.1. Each adrenal gland constitutes two distinct endocrine glands.



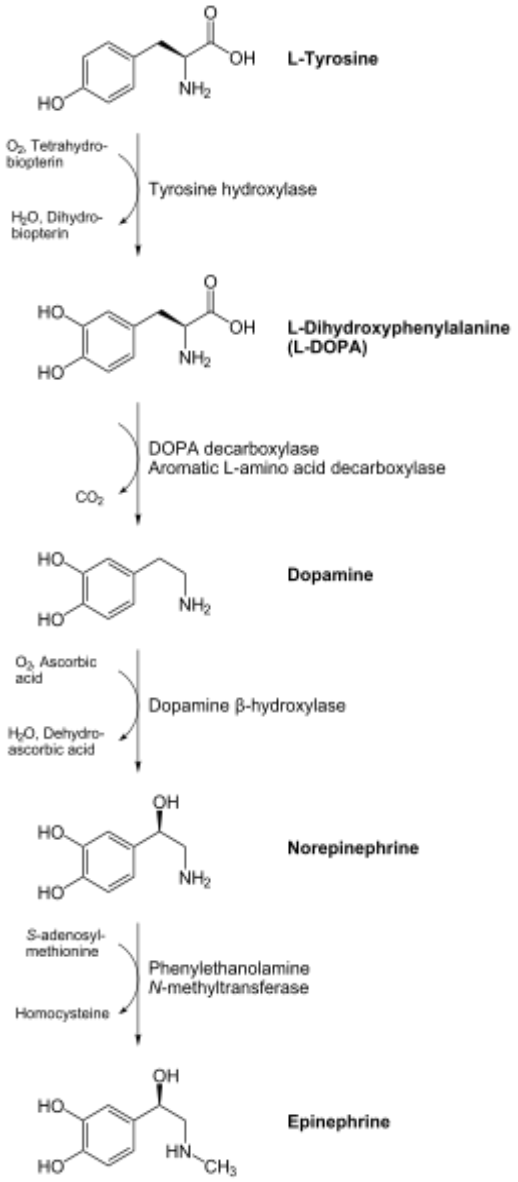
From http://www.britannica.com/EBchecked/topic-art/6405/121578/Human-adrenal-gland

Figure 7.1. Anatomy of the adrenal gland

The inner adrenal medulla mainly secretes the hormones adrenaline and noradrenaline. The surrounding adrenal cortex secretes different steroid hormones.

7.1.1. Adrenal medulla

The adrenal medulla, together with the sympathetic nervous system, is embryonically derived from neural crest cells. Adrenaline (about 75%) and noradrenaline (about 25%) are the main hormones secreted from the adrenal medulla. The main neurotransmitter between the sympathetic nervous system and the effector cells is noradrenaline.



The adrenal medulla synthesizes adrenaline (also called epinephrine) and noradrenaline (also called norepinephrine) from the amino acid phenylalanine through a series of enzymatic reactions – see Figure 7.2.

The regulation of adrenal release of adrenaline and noradrenaline is complex. Secretion is increased by adrenocorticotropic hormone (ACTH) from the anterior pituitary, by the adrenocortical hormone, cortisol, and by the sympathetic nervous system. The hormones inhibit their own secretion by decreasing the formation of the rate limiting enzyme tyrosine hydroxylase. There are other stimuli to adrenal medullary secretion.

The sympathetic nervous system and the adrenal medulla are usually activated during stress. The release of adrenaline and noradrenaline and the response of the body are often termed the “fight or flight” response. In general, the main effect of adrenaline and noradrenaline is to prepare the body for “fight of flight” by getting energy to the skeletal muscles. Blood will be shunted from viscera to skeletal muscles to provide glucose and oxygen to the muscles. The glucose concentration will increase through a variety of mechanisms. Heart rate and ventilation will increase to increase the oxygen supply.

From http://en.wikipedia.org/wiki/Epinephrine

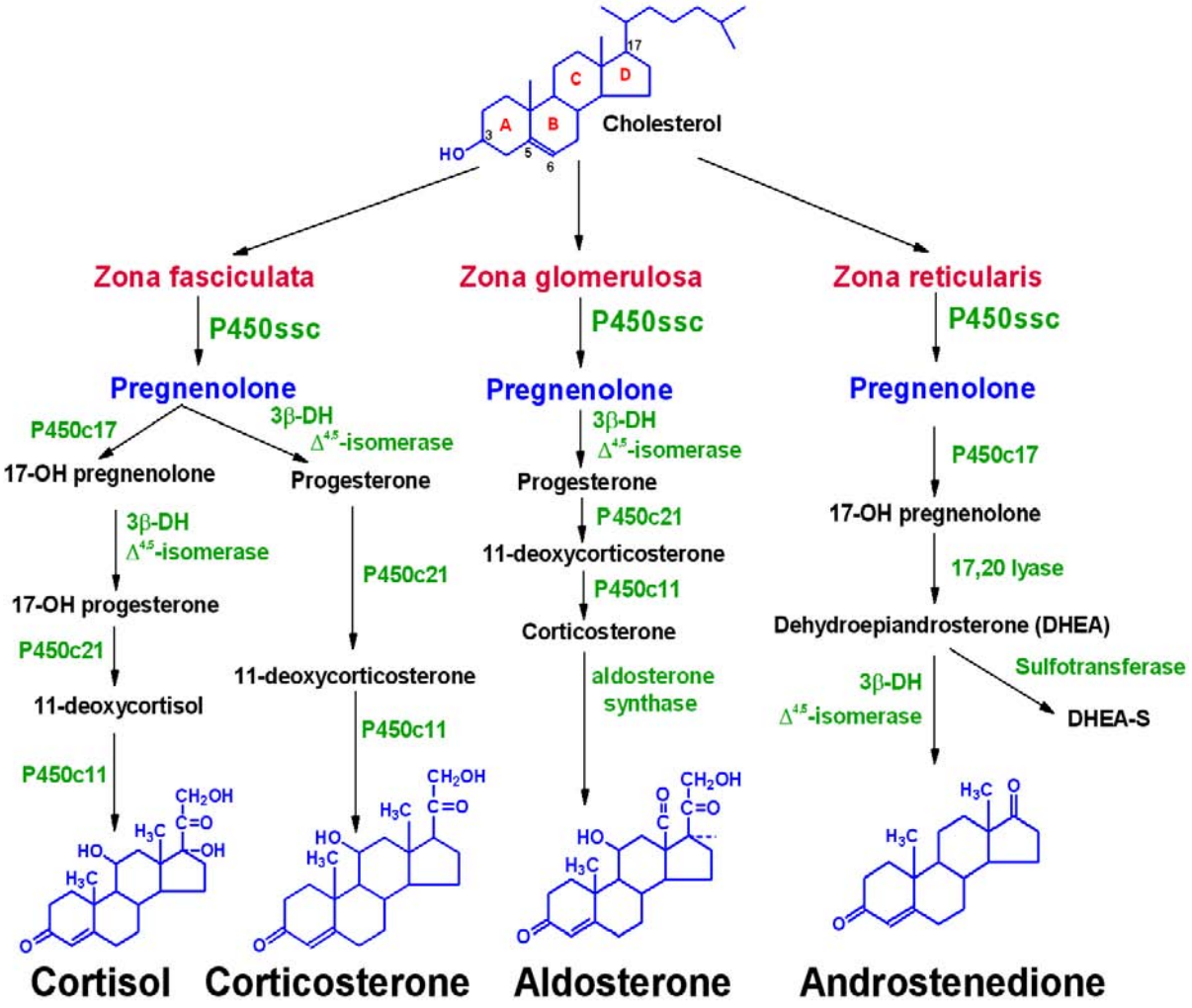
Figure 7.2. Biosynthesis of adrenaline

7.1.2. Adrenal cortex

The adrenal cortex secretes different steroid hormones. They are all synthesized from cholesterol – see Figure 7.3. Cortisol and corticosterone are glucocorticoids, which regulate carbohydrate metabolism. Aldosterone is a mineralocorticoid, which regulate the body levels of sodium and potassium. Androstenidione is an androgen, whose action is similar to that of testosterone although much weaker.

The adrenal cortex has insignificant stores of steroid hormones. Instead lipid droplets in the adrenal cells store cholesterol precursors, which can rapidly be metabolised to the steroid hormones. The level of the steroid hormones naturally varies during the day and night.

The adrenal cortex contains three different zones termed zona glomerulosa, zona fasciculate and zona reticularis – see Figure 7.1. The hormones are synthesized and secreted in different zones as can be seen on Figure 7.3 because the gland contains different enzymes (written in green in the figure) necessary for the synthesis in the different zones.



3b-DH is 3b-dehydrogenase, P450c11 is 11b-hydroxylase, P450c17 is 17a-hydroxylase, P450c21 is 21b-hydroxylase.
From http://eglobalmed.com/core/Biochemistry_Kings/web.indstate.edu/thcme/mwking/steroid-hormones.html

Figure 7.3. Synthesis of adrenal steroid hormones from cholesterol in humans

7.1.2.1. Species differences

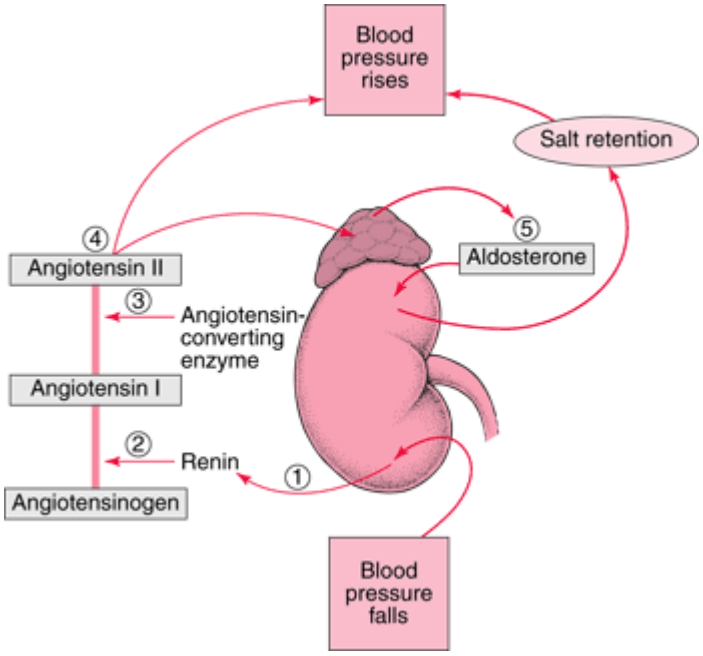
The zona reticularis in all animals is not always easily distinguishable and dedicated to androgen synthesis. In rodents, for instance, the zona reticularis also generates corticosterone,

which is the dominant glucocorticoid in rodents because of a negligible expression of P450c17. Female rodents also exhibit another cortical layer called the "X zone" whose function is not yet clear.

7.1.2.2. Zona glomerulosa (aldosterone secretion)

The primary role of aldosterone is to conserve sodium. This is accomplished by increasing the activity of the sodium pump of the epithelial cells especially in the kidney.

Aldosterone synthesis and secretion are regulated mainly by the renin-angiotensin system – see Figure 7.4. The renin-angiotensin system regulates blood pressure and water balance.



From http://www.merckmanuals.com/home/sec03/ch022/ch022a.html

When blood volume is low, the kidneys secrete renin ①.

Renin stimulates the production of angiotensin I ②, which is then converted to angiotensin II ③. Angiotensin II causes blood vessels to constrict, resulting in increased blood pressure. Angiotensin II also stimulates the secretion of aldosterone ④. Aldosterone causes the kidneys to increase the reabsorption of sodium and water into the blood ⑤. This increases the volume of fluid in the body, which also increases blood pressure.

Figure 7.4. The renin-angiotensin system

7.1.2.3. Zona fasciculate (cortisol and corticosterone secretion)

In humans cortisol is by far more important than corticosterone. Like adrenaline, cortisol is elevated during stress and increases the glucose concentration by various mechanisms. Cortisol also has anti-inflammatory and growth-suppressing effects.

The secretion of cortisol is regulated primarily by ACTH from the anterior pituitary via a negative feedback mechanism.

Secreted cortisol is transported in plasma bound to CBG (corticosteroid binding globulin).

7.1.2.4. Zona reticularis (androstenedione secretion)

Because androstenedione and other androgens secreted from the adrenal gland are much less potent than testosterone, they are of little physiological significance in men. Some of the weakly androgenic substances secreted by the adrenal gland may be converted to testosterone in peripheral tissue thus accounting for some androgenic effect initiated by the adrenal cortex. In women, sexual desire is probably more dependent upon androgens secreted by the adrenal glands than upon oestrogen.

ACTH from the anterior pituitary appears to be the major regulator of androgen secretion from the adrenal gland.

7.2. Establisment of CAGs for toxicity to the adrenal glands

7.2.1. CAG level 1: Toxicity to the adrenal glands

The active substances identified as having an effect on the adrenal glands in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 7.1.

Table 7.1. CAG level 1: Toxicity to the adrenal glands

2,4-D	Fosthiazate	Quizalofop-P (test substance: tefuryl)
Chlorothalonil	Iprodione	Tebuconazole
Chlorpyrifos-methyl	Lufenuron	Thiamethoxam
Epoxiconazole	Metconazole	Tralkoxydim
Fluoxastrobin	Oxadiazon	Triticonazole

7.2.2. CAG level 2: Phenomenological / specific effects on the adrenal glands

Various types of effects on the adrenal glands identified as a basis for establishing CAGs at level 2 include effects on the adrenal cortex such as enlargement, hypertrophy, hyperplasia, cytomegaly, vacuolation, lipid accumulation in adrenal cells, atrophy, degeneration, necrosis, and tumours.

Based on these effects, four distinct CAGs at level 2 are proposed. More information is given in Appendix D.

7.2.2.1. CAG level 2a: Hypertrophy / hyperplasia of the adrenal cortex

Hypertrophy is an increased size of cells, cytomegaly is an abnormal enlargement of cells, and hyperplasia is an increased number of cells. It is not always clear in the DARs, whether the terms ‘hypertrophy’ and ‘hyperplasia’ have been used to distinguish between the two different types of histopathological effects and the terms have in many cases been used synonymously.

For the purpose of the CAG project, gross pathology findings in form of enlarged zona(s) in the adrenal cortex and histopathological findings in form of hypertrophy, hyperplasia and

cytomegaly of cells in the adrenal cortex are allocated to a single CAG level 2, termed ‘CAG level 2a: Hypertrophy / hyperplasia of the adrenal cortex’.

The active substances identified as inducing one or more of the above-mentioned effects in the adrenal cortex are allocated to CAG level 2a and are listed in Table 7.2.

Table 7.2. CAG level 2a: Hypertrophy / hyperplasia of the adrenal cortex

2,4-D	Fosthiazate	Quizalofop-P (test substance: tefuryl)
Chlorothalonil	Iprodione	Tebuconazole
Chlorpyrifos-methyl	Lufenuron	

7.2.2.2. CAG level 2b: Fatty changes in the adrenal cortex

For some active substances, vacuolation and lipid accumulation is a description of the same effect in the DARs as the lipid is accumulated in the vacuoles. Although it is not clear from the DARs if this is always the case, these findings are, for the purpose of the CAG project, interpreted as representing the same type of effect in the adrenal cortex.

For the purpose of the CAG project, histopathological findings described as vacuolation, lipid droplets, lipid storage, fat/lipid vacuoles, fatty changes, fatty degeneration, and fatty metamorphosis are allocated to a single CAG level 2, termed ‘CAG level 2b: Fatty changes in the adrenal cortex’.

The active substances identified as inducing one or more of the above-mentioned effects in the adrenal cortex are allocated to CAG level 2b and are listed in Table 7.3.

Table 7.3. CAG level 2b: Fatty changes in the adrenal cortex

Chlorpyrifos-methyl	Iprodione	Tebuconazole
Epoxiconazole	Metconazole	Thiamethoxam
Fluoxastrobin	Oxadiazon	Tralkoxydim
Fosthiazate	Quizalofop-P (test substance: tefuryl)	Triticonazole

7.2.2.3. CAG level 2c: Cell degeneration / cell death in the adrenal cortex

Atrophy is loss of tissue, totally or partially and necrosis is death of cells and tissues.

For the purpose of the CAG project, histopathological findings described as atrophy, degeneration, and necrosis are allocated to a single CAG level 2, termed ‘CAG level 2c: ‘Cell degeneration / cell death in the adrenal cortex’.

The active substances identified as inducing one or more of the above-mentioned effects in the adrenal cortex are allocated to CAG level 2c and are listed in Table 7.4.

Table 7.4. CAG level 2c: Cell degeneration / cell death in the adrenal cortex

Epoxiconazole	Thiamethoxam	

7.2.2.4. CAG level 2d: Neoplasms in the adrenal cortex

One active substance was reported to induce adenomas in the adrenal cortex. This substance is allocated to the CAG level 2d and is listed in Table 7.5.

Table 7.5. CAG level 2d: Neoplasms in the adrenal cortex

Epoxiconazole		

7.2.2.5. Effects not considered relevant for CAGs at level 2

Effects such as sinus dilation, angiectasis (dilation of the blood vessels), pigmentation, multinucleated cells, amyloidosis (the tissue is filled with amyloid, a wax-like protein), mineralisation, and eosinophilic foci have been noted for a few active substances.

These effects are considered either as indirect effects on the adrenal gland or as being non-adverse effects and therefore, not relevant for CAGs at level 2 and consequently, not relevant in terms of CRA for effects on the adrenal glands.

7.2.3. CAG level 3: Mode of action

The most common effect noted in the adrenals is an increased relative adrenal weight for which a CAG is not considered to be relevant. An increased relative adrenal weight can be an indication of a toxic effect directly on the adrenal glands. If the secretion of e.g. cortisol is decreased because of a direct effect on the adrenal gland, the adrenal gland will try to increase the production and secretion of cortisol. The level of ACTH will increase and will stimulate the adrenal gland to grow in an attempt to maintain circulating basal levels of cortisol. In this situation, the level of ACTH will be increased and the level of cortisol will be low or normal.

However, an increased adrenal weight might also reflect an increased activity of the adrenal gland due to stress because of effects on other organ systems. In this situation the level of ACTH will also be increased, but the level of cortisol will at least initially also be increased.

Stress (and increased levels of cortisol) is known to induce thymus atrophy and decreased thymus weight. So for the active substances where thymus atrophy and/or decreased thymus

weight has been noted in addition to increased adrenal weight, the effect on the adrenal gland is most likely a consequence of stress due to effects on other organ systems and thus, the increased adrenal weight might be an indirect effect on the adrenal gland.

Standard toxicological studies are not designed to differentiate the effects of active substances on other organ systems from direct effects on the adrenal gland as the hormone levels in general are not measured.

Four types of phenomenological effects in the cortex of the adrenal gland have been noted: Hypertrophy / hyperplasia (CAG level 2a), fatty changes (CAG level 2b), cell degeneration / cell death (CAG level 2c), and neoplasms (CAG level 2d).

As for increased relative adrenal weight, the increased growth (hypertrophy) of the cortex can be stress-related or a direct toxic effect on the adrenal cortex.

If the production of steroid hormones is inhibited, the excess steroid precursors will accumulate in the cytoplasma and the number and size of cytoplasmic vacuoles may increase. However, hyperactivity in the adrenal gland because of stress may also result in vacuolation. For some of the active substances, vacuolation and lipid accumulation is a description of the same effect as the lipid is accumulated in the vacuoles.

Loss of adrenocortical cells (atrophy) due to necrosis, cell lysis, or apoptosis can be a consequence of severe or prolonged toxicity to the adrenal gland.

No information to clarify whether the phenomenological effects in the adrenal cortex are direct or indirect effects on the adrenal gland has been found, except for epoxiconazole, see below. No other information regarding the mode of action for these effects has been found. Consequently, none of the substances identified as having these phenomenological effects in the adrenal cortex can be allocated to a CAG level 3.

7.2.3.1. CAG level 3d1: Hormonal alterations

One substance, epoxiconazole, induced adenomas in the adrenal cortex and is allocated to CAG level 2d ‘Neoplasms in the adrenal cortex’. There is some information on the mode of action for the tumourigenic effect of epoxiconazole as adrenal hormone levels have been measured. The levels of androgen steroids and ACTH were increased while the levels of corticosterone and aldosterone were decreased. These changes can be explained by a test substance-related decrease of the adrenal enzyme activity of either 11- or 21-hydroxylase. The decreased adrenal steroid levels trigger a feedback response in the hypothalamic-pituitary axis resulting in increased ACTH levels. The continuous stimulation of adrenocortical cells by ACTH is considered to be responsible for the induction of the adrenal tumours by epoxiconazole. Based on this information, epoxiconazole is allocated to CAG level 3d1 ‘Hormonal alterations’ and is listed in Table 7.6.

Table 7.6. CAG level 3d1: Adenomas in the adrenal cortex related to hormonal alterations

Epoxiconazole		

7.2.4. CAG level 4: Mechanism of action

Effects on the adrenal glands can be elicited by a number of mechanisms:

- Inhibition of one or more of the enzymes of steroidogenesis (appears to be the most common mechanism) => excess steroid precursors => accumulation of increased cytoplasmic lipid
- Increased enzyme expression
- Enzymes in adrenal gland may metabolise and potentially activate chemicals
- Altered expression of receptors on the adrenocortical cells
- Inhibition of the StAR protein (Steroidogenic Acute Regulatory protein is a protein necessary for moving cholesterol from the cell cytosol to the inner mitochondrial membrane)
- Decreased binding affinity of CBG leading to increased plasma level of cortisol
- Increased metabolism of steroid hormones e.g. in the liver leading to decreased plasma levels
- Affected binding to target receptors
- Inhibition of hormones in the hypothalamus (CRH) or pituitary (ACTH)
- Generation of free radicals during steroid hydroxylation reactions
- Potential lipid peroxidation as a result of high membrane content of unsaturated fatty acids.

Mechanisms of action have not been studied for any of the active substances identified as having effects on the adrenal glands. Consequently, none of the substances identified as having effects on the adrenal gland can be allocated to a CAG level 4.

7.3. Discussion of CAGs for the adrenal glands

Fifteen active substances were identified to have effects on the adrenal glands and were allocated to CAG level 1. Four distinct CAGs at level 2 have been proposed. Information on mode of action is only available for one of the active substances, epoxiconazole. No information on the mechanism(s) of action is available for any of them. The information is summarised in Appendix F.

Epoxiconazole is the only active substance, which induces neoplasms and is the only active substance for which a mode of action has been proposed. It should be noted however, that the CAG level 2 for neoplasms is not recommended for CRA, see Chapter 4.

As no information regarding the mode / mechanism(s) of action for the phenomenological effects in the adrenal gland of the active substances in general has been found, the three CAGs at level 2 (2a-2c) could be considered for CRA for effects on the adrenal gland. It should be noted that the effects allocated to these CAGs may be interrelated.

7.4. Recommended CAGs for the adrenal glands

The following CAGs at level 2 are recommended for CRA for effects on the adrenal gland:

- CAG level 2a: Hypertrophy / hyperplasia of the adrenal cortex, see Table 7.2.
- CAG level 2b: Fatty changes in the adrenal cortex, see Table 7.3.
- CAG level 2c: Cell degeneration / cell death in the adrenal cortex, see Table 7.4.

8. Bone marrow

8.1. Introduction

The bone marrow, also called myeloid tissue, is confined to the cavities of bones. Bone marrow consists of blood vessels, nerves, mononuclear phagocytes, stem cells, blood cells in various stages of differentiation, and fatty tissue.

- Adults have two kinds of bone marrow:
- Red marrow (active marrow, consisting mainly of haematopoietic tissue)
- Yellow marrow (inactive marrow, consisting mainly of fat cells – it is the large quantities of fat that makes it yellow).

Not all bones contain active marrow. In adults, active marrow exists in the pelvic bones (ring formed of the two hip bones), vertebrae, cranium and mandible (lower bone in the jar), sternum (the breastbone) and ribs, and extreme proximal portions of the humerus (top bone in the arm) and femur (top bone in the leg). Inactive marrow predominates in cavities of other bones.

The bone marrow contains three types of stem cells:

- Haematopoietic stem cells that give rise to the three classes of blood cells that are found in the circulation: White blood cells (leukocytes), red blood cells (erythrocytes), and platelets (thrombocytes).
- Mesenchymal stem cells that have the capability to differentiate into osteoblasts, chondrocytes, myocytes, and many other types of cells.
- Endothelial stem cells.

Blood cell production, termed haematopoiesis, occurs under normal conditions only in the bone marrow and is known as medullary haematopoiesis. Medullary haematopoiesis increases in response to various disorders that deplete blood cells. Increased medullary haematopoiesis is thus, an indirect effect on the bone marrow, i.e., is secondary to a decreased number / depletion of circulating blood cells, see Chapter 14 ‘Haematological system’.

The blood vessels in the bone marrow constitute a barrier, the bone marrow barrier, which inhibits immature blood cells from leaving the bone marrow. Only mature blood cells contain the membrane proteins required to attach to and pass the bone marrow barrier.

The normal bone marrow architecture can be displaced by malignancies, aplastic anaemia, or infections such as tuberculosis, leading to a decrease in the production of blood cells and platelets. In addition, cancers of the haematological progenitor cells in the bone marrow can arise; these are the leukemias.

8.2. Establishment of CAGs for toxicity to the bone marrow

8.2.1. CAG level 1: Toxicity to the bone marrow

Various types of effects on the bone marrow were identified including:

- Hyperplasia
- Hypercellularity
- Increased medullary haematopoiesis
- Increased number of megakaryocytes
- Haemosiderosis (increased amounts of a yellow-brown iron containing pigment)
- Hypoplasia
- Hypocellularity
- Atrophy
- Necrosis
- Haemorrhage
- Congestion

Most of the active substances identified to affect the bone marrow induced hyperplasia, hypercellularity, increased medullary haematopoiesis, increased number of megakaryocytes, haemosiderosis, effects that are secondary to a direct effect on the cellular elements in the blood stream and are therefore covered by the CAGs for the cellular elements in the blood stream, see Chapter 14. Overall, these effects are not considered relevant in terms of CRA for direct effects on the bone marrow and the active substances causing these effects are therefore not considered further for CAGs in relation to the bone marrow.

Some of the active substances identified to affect the bone marrow induced hypocellurarity, hypoplasia, atrophy and/or necrosis, effects that are considered as a direct effect on the bone marrow. The active substances identified as causing these effects in the bone marrow in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 8.1.

Table 8.1. CAG level 1: Toxicity to the bone marrow

2,4-D	Clodinafop	Metiram
2,4-D metabolite: 2,4-dichlorophenol	Clothianidin	Pethoxamid
Acibenzolar-S-methyl metabolite: CGA 210007	Fluopicolide	Pyraflufen-ethyl
Bifenazate	Flutolanil	Spinosad
Bromoxynil	Ioxynil	Trifloxystrobin
Chlorotoluron	Isoproturon	Tritosulfuron-methyl
Chlorsulfuron metabolite: IN-A4097	MCPA og MCPB	

8.2.2. CAG level 2: Phenomenological / specific effects on the bone marrow

Based on the effects on the bone marrow considered as being direct effects, two distinct CAGs level 2 are proposed. More information is given in Appendix F.

8.2.2.1. CAG level 2a: Hypoplasia

Hypoplasia is a decrease in the number of cells and hypocellularity is an abnormal decrease in the number of cells.

For the purpose of the CAG project, hypoplasia and hypocellularity in the bone marrow are allocated to a single CAG level 2, termed ‘CAG level 2a: Hypoplasia’.

The active substances identified as inducing hypoplasia or hypocellularity in the bone marrow are allocated to CAG level 2a and listed in Table 8.2.

Table 8.2. CAG level 2a: Hypoplasia

2,4-D	Clodinafop	Metiram
Acibenzolar-S-methyl metabolite: CGA 210007	Clothianidin	Pyraflufen-ethyl
Bifenazate	Fluopicolide	Spinosad
Bromoxynil	Flutolanil	Trifloxystrobin
Chlorotoluron	Ioxynil	Tritosulfuron-methyl
Chlorsulfuron metabolite: IN-A4097	Isoproturon	

8.2.2.2. CAG level 2b: Cell degeneration / cell death

Atrophy is loss of tissues, totally or partially and necrosis is death of cells and tissues.

For the purpose of the CAG project, histopathological findings described as atrophy and necrosis are allocated to a single CAG level 2, termed ‘CAG level 2b: ‘Cell degeneration / cell death’.

The active substances identified as inducing one or more of the above-mentioned effects in the bone marrow are allocated to CAG level 2b and are listed in Table 8.3.

Table 8.3. CAG level 2b: Cell degeneration / cell death

2,4-D metabolite: 2,4-dichlorophenol	Pethoxamid	Trifloxystrobin
MCPA and MCPB	Spinosad	

8.2.2.3. Effects not considered relevant for CAGs at level 2

Effects such as discolouration, haemorrhage and congestion of the bone marrow have been observed. These effects are considered as being non-adverse or non-specific effects and therefore, not relevant for CAGs at level 2 and consequently, not relevant in terms of CRA for effects on the bone marrow.

8.2.3. CAG level 3: Mode of action

For some of the effects considered as being direct effects on the bone marrow described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

8.2.3.1. CAG level 3a1: Hypoplasia related to a direct effect on the bone marrow

For a metabolite of the active substance acibenzolar-S-methyl (metabolite CGA 210007) identified as inducing hypoplasia in the bone marrow and allocated to CAG level 2a, there is information in the DAR that the effect is probably due to a direct cytotoxic effect of the metabolite to bone marrow cells. This substance is allocated to CAG level 3a1 and is listed in Table 8.4.

Table 8.4. CAG level 3a1: Hypoplasia related to a direct effect on the bone marrow

Acibenzolar-S-methyl (metabolite CGA 210007)		

8.2.3.2. CAG level 3b1: Atrophy related to a direct effect on the bone marrow

For two (MCPA and MCPB – MCPA a metabolite of MCPB) of the three active substances identified as inducing atrophy in the bone marrow and allocated to CAG level 2b, there is information in the respective DARs that the effect is probably due to a direct effect on the bone marrow. These substances are allocated to CAG level 3b1 and are listed in Table 8.5.

Table 8.5. CAG level 3b1: Atrophy related to a direct effect on the bone marrow

MCPA and MCPB		

8.2.4. CAG level 4: Mechanism of action

For three active substances, a mode of action for direct effects observed in the bone marrow has been proposed. However, no information regarding the mechanism(s) of action has been found for these four substances. Consequently, these four substances cannot be allocated to a CAG level 4.

No information on mechanism(s) of action has been found for any of the other active substances identified as having an effect on the bone marrow.

8.3. Discussion of CAGs for the bone marrow

Twenty active substances or metabolites were identified to have potential direct effects on the bone marrow and were allocated to CAG level 1. Two distinct CAGs at level 2 have been proposed. Information on mode of action is available for three of the active substances and information on the mechanism(s) of action is available for a few of them. The information is summarised in Appendix G.

A mode of action for direct effects on the bone marrow has been identified for one of the substances allocated to CAG level 2a, and for two of the substances allocated to CAG level 2b. There is, however, no information on the mechanism(s) of action for any of them. Therefore, the two CAGs at level 3 could be considered for CRA. However, as only one active substance is allocated to each of these CAGs at level 3 (MCPB allocated to CAG level 3b1 is extensively metabolised to MCPA in mammalian species and MCPA is the toxic metabolite of MCPB), these two CAGs at level 3 are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active substances justifies the mode of action forming the basis for the respective CAGs.

No information regarding the mode of action for the remaining active substances allocated to CAG level 2a and 2b, respectively, has been found. Therefore, these CAGs at level 2 could be considered for CRA.

8.4. Recommended CAGs for the bone marrow

The following CAGs at level 2 are recommended for CRA for effects on the bone marrow:

- CAG level 2a: Hypoplasia, see Table 8.2.
- CAG level 2b: Cell degeneration / cell death, see Table 8.3.

The following CAGs are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active

substances justifies the mode of action / reveals the phenomenological effects forming the basis for the respective CAGs:

- CAG level 3a1: Hypoplasia related to a direct effect on the bone marrow, see Table 8.4.
- CAG level 3b1: Atrophy related to a direct effect on the bone marrow, see Table 8.5.

9. Bones / skeleton

9.1. Introduction

Bones are the calcified pieces of connective tissue which make the skeleton. The human skeleton consists of 206 bones. The major functions of the bones are:

- to give form to the body, permit movement and to provide protection of vital organs
- to house the bone marrow which produces blood cells
- to store minerals such as calcium, phosphate, magnesium

Bones are composed of:

- bone cells (osteoblasts, osteocytes, and osteoclasts)
- bone matrix (mainly collagen, proteoglycans, and glycoproteins)
- bone minerals (mainly calcium and phosphate)
- blood vessels and nerves

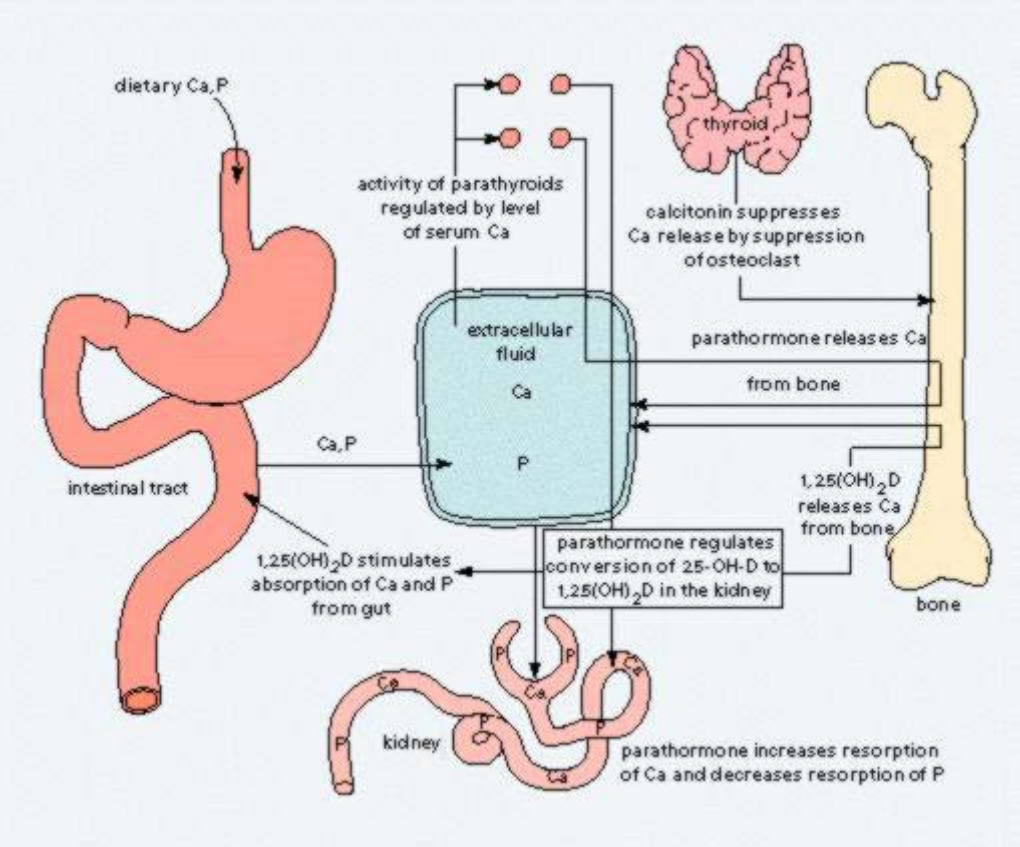
Osteoblasts are the bone-forming cells. They are responsible for bone matrix synthesis and its subsequent mineralization.

Osteocytes are osteoblasts that become incorporated within the newly formed bone matrix. They have a role in maintaining the bone matrix.

Osteoclasts function primarily to resorb (remove) bone during processes of growth and repair.

The hardness and rigidity of bone is due to the presence of minerals in the bone matrix. As can be seen from figure 9.1 the metabolism of calcium and phosphate is regulated by a complex interplay mainly between the parathyroid hormone, vitamin D and calcitonine. Acting together, these substances determine the amount of dietary calcium and phosphate absorbed from the intestine, the reabsorption and excretion of calcium and phosphate by the kidney, and the deposition and release of calcium and phosphate from the bone. Both parathyroid hormone and active vitamin D stimulate osteoclasts to resorb bone (and release calcium and phosphate from bone) whereas calcitonin suppresses calcium release from bone by suppression of osteoclasts.

The production of parathyroid hormone, active vitamin D and calcitonin is mainly regulated by the serum level of calcium but also by other minerals and other substances.



From http://www.orthoteers.com/(S(j0xys0d0pf4cams5k0qeo11b))/mainpage.aspx?section=10&article=48

Figure 9.1. Calcium and phosphate metabolism

9.2. Establishment of CAGs for toxicity to the bones / skeleton

9.2.1. CAG level 1: Toxicity to the bones / skeleton

The active substances identified as having an effect on the bones / skeleton in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 9.1.

Table 9.1. CAG level 1: Toxicity to the bones / skeleton

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Flazasulfuron	Thiophanate-methyl
Cinidon ethyl	Sulfosulfuron	Tolylfluanid

Etoxazole	Tetraconazole	

It should be noted that developmental effects on bones / skeleton, such as delayed ossification and skeletal malformations in foetuses, which are generally observed in reproductive toxicity and teratogenicity studies are not included here, but considered in the section on effects on the reproductive system and developmental toxicity (chapter 25).

9.2.2. CAG level 2: Phenomenological / specific effects on bones / skeleton

Various types of effects on bones / skeleton were identified as a basis for establishing CAGs at level 2. Based on these effects, three distinct CAGs at level 2 are proposed. More information is given in Appendix H.

9.2.2.1. CAG level 2a: Fibrous osteodystrophy

Fibrous osteodystrophy is a lesion of the bone in which fibrous tissue replaces resorbed bone.

The active substances identified as inducing fibrous osteodystrophy are allocated to CAG level 2a and are listed in Table 9.2.

For thiophanate-methyl, the effect on bone was described in the DAR as demineralisation of bone. However, as fibrous osteodystrophy is a consequence of demineralisation of bone, thiophanate-methyl was also allocated to the CAG level 2a.

Table 9.2. CAG level 2a: Fibrous osteodystrophy

2-Phenylphenol	Flazasulfuron	Thiophanate-methyl
Cinidon ethyl	Sulfosulfuron	

9.2.2.2. CAG level 2b: Hypertrophy / hyperplasia

Active substances that induce effects described as hyperostosis (excessive growth of bone), hyperplasia of bone tissue (increased number of cells in bone), osseous hypertrophy (increased size of cells in bone), and thickening of bone are allocated to CAG level 2b and are listed in Table 9.3.

Table 9.3. CAG level 2b: Hypertrophy / hyperplasia

Etoxazole	Tetraconazole	Tolylfluanid

9.2.2.3. CAG level 2c: Osteopetrosis.

One active substance was identified as inducing osteopetrosis (hardening of bone). This substance is allocated to CAG level 2c and is listed in Table 9.4.

Table 9.4. CAG level 2c: Osteopetrosis

Tolylfluanid		

9.2.3. CAG level 3: Mode of action

For some of the phenomenological / specific effects on the bones / skeleton described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

9.2.3.1. CAG level 3a1: Chronic nephropathy and hyperparathyroidism

In early chronic renal failure, excreted phosphate levels decrease and the plasma phosphate concentration increases. Plasma phosphate binds calcium which results in hypocalcaemia. As a consequence, the parathyroids start to secrete parathyroid hormone in order to return calcium and phosphate levels to normal. The consequence is activation of osteoclasts and resorption of bone. At later stages of chronic renal failure the effects on bone are accelerated as the synthesis of active vitamin D may be impaired.

For four of the five active substances identified as inducing fibrous osteodystrophy and allocated to CAG level 2a, the mode of action was suggested to be secondary to chronic nephropathy and hyperparathyroidism. These substances are allocated to CAG level 3a1 and are listed in Table 9.5.

Table 9.5. CAG level 3a1: Fibrous osteodystrophy related to chronic nephropathy and hyperparathyroidism

Cinidon ethyl	Sulfosulfuron	Thiophanate-methyl
Flazasulfuron		

9.2.3.2. CAG level 3b1: Accumulation of fluoride

For two of the three active substances (tetraconazole and tolylfluanid) identified as inducing hypertrophy / hyperplasia and allocated to CAG level 2b, the mode of action has been ascribed to accumulation of fluoride in bone. Fluoride is released when the substances are metabolised in the liver. For the remaining substance (etoxazole) allocated to CAG level 2b, the mode of

action is likely the same, as etoxazole like tetraconazole and tolylfluanid contains fluoride. These substances are allocated to CAG level 3b1 and are listed in Table 9.6.

Table 9.6. CAG level 3b1: Hypertrophy / hyperplasia of bone related to accumulation of fluoride in bone

Etoxazole	Tetraconazole	Tolylfluanid

Whitening of bone is caused by an increased concentration of fluoride in the bone, an effect covered by the CAG level 3b1 for accumulation of fluoride in bone.

9.2.4. CAG level 4: Mechanism of action

No information on mechanism(s) of action have been found for any of the active substances identified as having an effect on the bones / skeleton.

9.3. Discussion of CAGs for the bones / skeleton

Eight active substances were identified to have effects on the bones / skeleton and were allocated to CAG level 1. Three distinct CAGs at level 2 have been proposed. Information on mode of action is available for some of the active substances. No information on the mechanism(s) of action is available for any of them. The information is summarised in Appendix I.

For four of the five active substances allocated to CAG level 2a, the available information on mode of action indicates that the effects are indirect effects in the bone, i.e., are secondary to chronic nephropathy and hyperparathyroidism (CAG level 3a1). The CAG level 3a1 as well as the CAG level 2a for these substances are therefore, not considered relevant in terms of CRA for a direct effect on the bones / skeleton.

For the three active substances allocated to CAG level 2b, the available information on mode of action indicates that the effects are due to accumulation of fluoride in bone (CAG level 3b1), which is released when the substances are metabolised in the liver. This is considered as being a direct effect of these substances on bone and therefore, relevant for CRA.

No information regarding the mode of action for the remaining active substance allocated to CAG level 2a as well as for the active substance allocated to CAG level 2c has been found. Therefore, these two CAGs at level 2 could be considered for CRA. However, as only one active substance remains allocated to CAG level 2a and only one substance is allocated to CAG level 2c, these two CAGs at level 2 are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active substances reveals the phenomenological effects forming the basis for the respective CAGs and for CAG level 2a, provided that no information on the mode of action is available.

9.4. Recommended CAGs for bones / skeleton

The following CAG at level 3 is recommended for CRA for effects on the bones / skeleton:

- CAG level 3b1: Accumulation of fluoride, see Table 9.6.

The following CAGs are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active substances reveals the phenomenological effects forming the basis for the respective CAGs:

- CAG level 2a: Fibrous osteodystrophy, see Table 9.2 (only if no information on the mode of action is available).
- CAG level 2c: Osteopetrosis, see Table 9.4.

10. Cardiovascular system

10.1. Introduction

The cardiovascular (CV) system has two units: the heart (myocardium) and the vascular system (vasculature comprising arteries, capillaries and veins, lymphatic system; vascular bed). The function of both units is to supply cells of all tissues with nutrients, oxygen, hormones and metabolites and removing "waste products" from tissue. Thus the CV system plays an important role in maintaining homeostasis in the body, including maintaining body temperature. Damage to the CV system by a toxicant may impact on other organs, especially highly vascularised organs that are dependent on nutrients and oxygen carried by the blood.

The assessment of the potential toxic effects of a pesticide active substances on the CV system may involve both a review of the results from routine regulatory toxicity studies and of special studies designed to detect changes in function and structure of the heart and vascular bed.

The majority of animal studies on pestide active substances have been conducted in rodents and were not designed to detect clinically manifested disturbances in the function of the heart and/or blood vessels, although they can show a range of clinical, biochemical and pathological effects. Furthermore the routine regulatory toxicity studies will not provide information on potential atherogenicity or acceleration of atherosclerosis by test compounds because laboratory rodents and dogs do not develop atherosclerosis.

10.1.1. Manifestation of CV toxicity

CV toxicity is manifested directly on the CV system or indirectly on other organs, which are dependent on nutrient and oxygen carried by the blood. Both categories are manifested by functional changes (alterations) often clinically manifested or by morphological changes which are recorded by macroscopy and/or microscopy (morphological changes are also termed structural alterations, (histo)pathological changes), and changes in absolute and/or relative organ weights.

Functional changes (alterations) to the heart induced by direct action of a toxicant are manifested as disturbed rhythmicity and contractility of the heart (arrhythmias).

Morphological changes (structural alterations) may lead to functional changes, which persists after cessation of the exposure to the toxicant.

The range of reactions to a toxicant in blood vessels is limited. The pathogenesis of any change may be difficult to determine by morphological examination. Functional changes such as prolonged vasoconstriction may result in ischemia and necrosis of the surrounding tissues with no obvious morphological change in the vessel.

Vascular injuries may be due to a direct (specific) interaction with the compound or it may be a secondary (indirect; unspecific) effect.. Secondary lesions develop as an extension of a disease process in surrounding tissues. However, direct vascular diseases (toxicoses) are the most frequent type seen and the lesions may be generalized or regional in distribution.

Blood vessels vary in their functional responses to chemicals. The heterogeneity in response occurs not only between the vein and arteries, but between anatomically similar vessels in different regions of the circulation.

10.2. Establishment of CAGs for toxicity to the cardiovascular system

The CAG level system constructed in order to establish CAGs for CV toxicity is based on the anatomical components of the CV system: Heart, vascular bed (major vessels and peripheral capillaries, and vasculature of organs other than the heart. The CAGs takes into account the clinical/functional and morphological changes noted after exposure to the active substances. In the evaluation, special attention was paid to determine whether the toxic effects on the CV system were due to a direct (specific) interaction of the compound with the CV system. If the changes seen were considered to be secondary to some other effects, i.e. indirect (unspecific), the compound was not included in a CAG.

10.2.1. CAG level 1: Toxicity to the cardiovascular system

The active substances identified as having an effect on the cardiovascular system in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 10.1.

Table 10.1. CAG level 1: Toxicity to the cardiovascular system

Chlorsulfuron	Difenoconazole	Prosulfuron
Chlorsulfuron, metabolite IN-A4098	Formetanate	Quizalofop-P-tefuryl
Clothianidin	Fuberidazole	Spinosad
Cyflufenamid	Glufosinate	Tepraloxydim
Cyromazine	Propaquizafop	Ziram

10.2.2. CAG level 2: Phenomenological / specific effects on the CV system

Various types of effects on the CV system were identified as a basis for establishing CAGs at level 2:

- Functional changes in the heart
- Morphological changes in the hearth
- Functional changes in the vascular bed
- Morphological changes in the vascular bed
- Toxicity to the vasculature of different organs

Based on these effects, five distinct CAGs at level 2 are proposed.

10.2.2.1. CAG level 2a: Functional changes in the heart

The functional changes (clinically manifested disturbances) reported were abnormal heart rate (bradycardia, tachycardia) and arrhythmia, either supraventricular (supraventricular tachycardis, arterial fibrillation) or ventricular (ventricular fibrillation, ectopic heart beat, heart block).

These functional effects are difficult to detect by a clinical examination in routine regulatory studies in rodents. They can be detected in special studies, for instance when electrocardiography is performed.

Functional symptoms from the hearth caused by acetylcholine esterase inhibitors will occur together with several other effects characteristic for overstimulation of muscarinic receptors.

More detailed information is given in Appendix J.

The active substances identified as inducing functional changes in the heart are allocated to CAG level 2a and are listed in Table 10.2.

Table 10.2. CAG level 2a: Functional changes in the heart

Clothianidin	Fuberidazole	Glufosinate
Cyflufenamid		

10.2.2.2. CAG level 2b: Morphological changes in the heart

The morphological changes observed were cardiac hypertrophy, cardiac atrophy, dilated cardiomyopathies, degenerations (hydropic, myofibrillar, fatty (lipofuscinosis), necrosis, inflammation (myocarditis, infarction), and mineralisation (predominantly calcification). The morphological changes can be detected in toxicological studies, in which histological examinations are performed. More detailed information is given in Appendix K.

The active substances identified as inducing morphological changes in the heart are allocated to CAG level 2b and are listed in Table 10.3.

Table 10.3. CAG level 2b: Morphological changes in the heart

Chlorsulfuron, and metabolite IN A4098	Formetanate	Prosulfuron
Clothianidin	Fuberidazole	Quizalofop-P-tefuryl
Cyflufenamid	Glufosinate	Spinosad
Difenoconazole	Propaquizafop	Tepraloxydim

10.2.2.3. CAG level 2c: Functional changes in the vascular bed

The functional changes are clinically manifested disturbances in the function of blood vessels. The functional changes in blood vessels (arteries and veins) like hypo- or hypertension (as a consequence of vasodilatation or vasoconstriction) cannot be detected in the clinical phase of the routine regulatory studies in rodents. They can be detected in special *in vivo* studies, in which measurement of blood pressure, blood flow measurement, or a direct observation of blood vessels is performed. More detailed information is given in Appendix L.

The active substances identified as inducing functional changes in the vascular bed are allocated to CAG level 2c and are listed in Table 10.4.

Table 10.4. CAG level 2c: Functional changes in the vascular bed

Fuberidazole	Spinosad	Tepraloxydim

10.2.2.4. CAG level 2d: Morphological changes in the vascular bed

The morphological changes in the wall of major blood vessels (arteries and veins) or peripheral capillaries can be detected in routine regulatory toxicity studies which include gross examination of the large vessels and histopathological examination of blood vessels.

Only one active substance was identified to cause morphological chandes in the blood vessels (endothelial cells). It produced haemorrhage possibly due to damage of the wall of the blood vessels. This substance is allocated to CAG level 2d and is listed in Table 10.5.

Table 10.5. CAG level 2d: Morphological changes in the vascular bed

Quizalofop-P-tefuryl		

10.2.2.5. CAG level 2e: Toxicity to the vasculature of different organs

The most frequent morphological change in the vasculature of different organs after exposure to the active substances was congestion. The aetiology of congestion can be connected to cardiac toxicity (diminished “vital power” of the heart) i.e. pulmonary congestion or to obstruction of escape of blood from the organ in question due to functional or morphological changes. Other vascular changes in different organs, which may be recorded, are for instance an infarct, haemorrhage, thrombus, vascular ectasia or the expansion of sinusoids in endocrine tissue. The two last changes however, may be a part of the physiology of aging laboratory animal, thus not associated with toxicity toward vasculature of the organ in question.

The changes in vasculature of certain organs can be detected in routine regulatory toxicity studies which include gross and histopathological examination of organs. Their specificity towards the CV system can be unclear as these changes can be secondary lesions as an extension of a direct toxicity toward a specific organ.

More detailed information is given in Appendix M.

The active substances considered to exert direct toxicity of the vasculature in different organs (congestion / haemorrhage) are allocated to CAG level 2e and are listed in Table 10.6.

Table 10.6. CAG level 2e: Toxicity to the vasculature of different organs

Clothianidin	Formetanate	Spinosad
Cyflufenamid	Glufosinate	Ziram
Cyromazine	Quizalofop-P-tefuryl	

10.2.2.6. Effects not considered relevant for CAGs at level 2

Changes in the relative weight of the heart are considered to be indirect and unspecific to the heart and therefore, not considered relevant for a CAG at level 2 (see also Chapter 4).

10.2.3. CAG level 3 and level 4: Mode / mechanism of action

No consistent modes/mechanisms of actions could be identified that would form the basis for CAGs at level 3 or 4 for toxicity on the CV system.

The modes/mechanisms of action behind the functional changes in the heart could be related to either disturbances in ion transport or disturbances in the contractile or energy producing systems causing the following effects: Chronotropic (on heart rate), inotropic (on contractility), dromotropic (on conductivity), bathmotropic (on excitability). Disturbances due to inhibition of acetylcholinesterase (signs of stimulation of muscarinic receptor) were also reported.

Biochemical mechanisms behind the morphological changes could be: Hypoxia, oxidative stress, mitochondrial dysfunction, and inhibition of mitochondrial enzymes.

10.3. Discussion of CAGs for the cardiovascular system

Twenty-two active substances were identified to have effects on the cardiovascular system and were allocated to CAG level 1. Five distinct CAGs at level 2 have been proposed. No information on the modes/mechanisms of action is available for any of them. The information is summarised in Appendix N.

Most of the substances that showed effects related to the CV system produced functional (CAG level 2a) or morphological (CAG level 2b) changes in the heart. These are the only two CAGs suggested to be considered for CRA for effects on the heart.

The functional changes (CAG level 2c) in the vascular bed (major vessels) were general or visceral congestion or congestion in capillaries, decreased blood pressure and circulatory failure. No specific modes of action have been demonstrated, but these effects could have occurred due to vasodilatation, which can be caused by relaxation of smooth muscles of the blood vessel wall.

Congestion (CAG level 2e) was also the most often recorded sign of toxicity towards the vasculature of specific organs. Haemorrhage was another often recorded change in this CAG. Haemorrhage is a consequence of the compromised wall of a blood vessel. In most cases, the haemorrhage is caused by a direct effect of the toxicant on the wall (endothelial cells) of the blood vessel (due to its presence in the blood).

10.4. Recommended CAGs for the cardiovascular system

The following CAGs at level 2 are recommended for CRA for effects on the cardiovascular system:

- CAG level 2a: Functional changes in the heart, see Table 10.2.
- CAG level 2b: Morphological changes in the heart, see Table 10.3.
- CAG level 2c: Functional changes in the vascular bed, see Table 10.4.
- CAG level 2e: Toxicity to the vasculature of different organs, see Table 10.6.

The following CAG is not recommended for CRA for the time being. However, the CAG may become relevant in the future provided that new information on other active substances reveals the phenomenological effect forming the basis for the CAG:

- CAG level 2d: Morphological changes in the vascular bed, see Table 10.5.

11. Eye

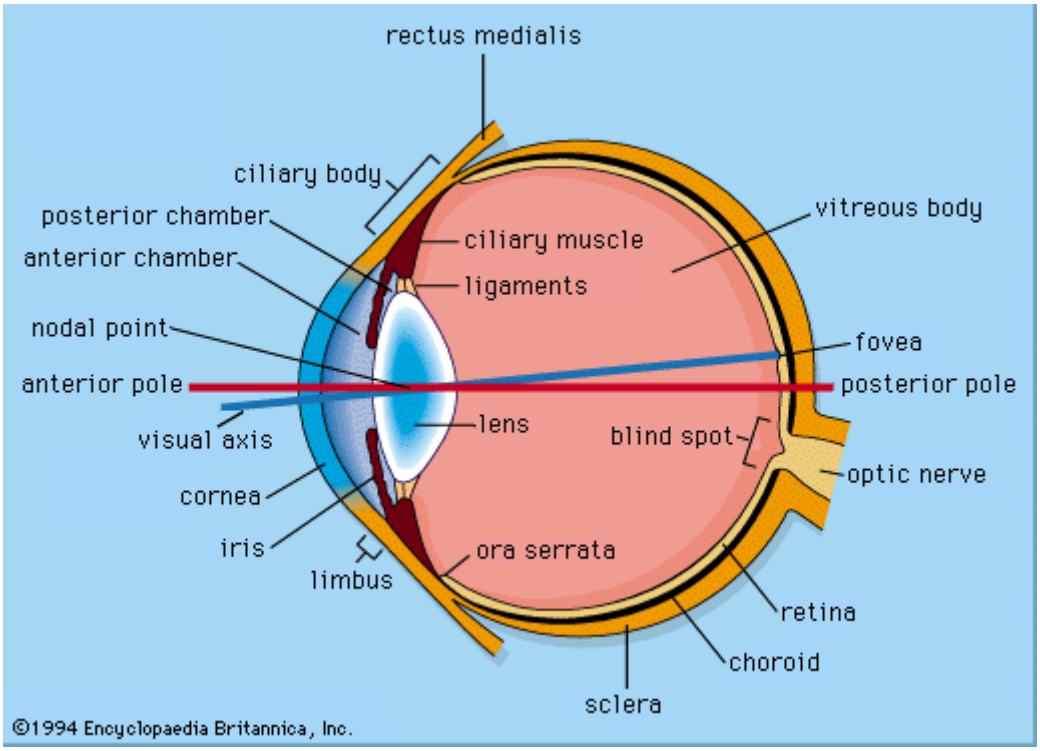
11.1. Introduction

The eyes are complex sense organs responsible for vision. Each eye has receptors, a lens system for focusing light on the receptors, and a system of nerves for conducting impulses from the receptors to the brain.

The eye is protected by external structures consisting of the eyelids, conjunctivae, and lacrimal apparatus. The eyeball is the receptor part of the eye, a round ball of tissue through which light passes and which is controlled by various muscles. The eyeball is formed of three layers (sclera, choroid and retina), which encloses the aqueous humour, lens and vitreous body (Figure 11.1):

- The sclera is the thick, white, outermost layer which becomes transparent at the cornea, the part of the sclera in the central anterior region that allows light to enter the eye.
- The uvea is formed of the iris, the ciliary body and choroid. The choroid is the deeply pigmented middle layer that prevents light from scattering inside the eye. The iris, which is connected to the choroid by the ciliary body, has a round opening, the pupil, through which light passes. The ciliary muscle changes the shape of the lens in order to focus on objects at different distances.
- The retina is the innermost layer and contains millions of rods and cones, which are special photoreceptors that convert the ocular light image into nerve impulses before sending them toward the brain. The photoreceptive rods and cones are distributed over the entire retina, except where the optic nerve leaves the eyeball.
- The aqueous humour is the clear fluid filling the cavity between the cornea and the lens.
- The lens is the part of the eye behind the iris and pupil which focuses light coming from the cornea onto the retina. The lens is an avascular, transparent tissue surrounded by an elastic, acellular, collagenous capsule, and is composed of only a single cell type. It can be arbitrarily divided into its anterior (corneal side) and posterior parts.
- The vitreous body is the transparent jelly filling the main behind the lens.

The anterior / posterior chambers of the eye are parts of the aqueous chamber. The anterior chamber is the space between the cornea and the iris, while the posterior chamber is the much smaller space behind the iris.



From http://www.britannica.com/EBchecked/topic/1688997/human-eye

Figure 11.1. Anatomy of the eye

11.2. Establishment of CAGs for toxicity to the eye

11.2.1. CAG level 1: Toxicity to the eye

The active substances identified as affecting the eye in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 11.1.

Table 11.1. CAG level 1: Toxicity to the eyes

2,4-D	Famoxadone	Metribuzin
2,4-DB	Fenhexamid	Oxamyl
2-Phenylphenol	Fenpropidin	Oxasulfuron
Acetamiprid	Flazasulfuron	Penconazole
Benfluralin	Fluazinam	Propamocarb
Chlorothalonil (metabolite: SDS-3701)	Flufenacet (formerly fluthiamide)	Propineb (metabolite: PU)
Chlorpropham	Fosthiazate	Prosulfocarb
Chlorpyrifos	Glufosinate	Prothioconazole
Cymoxanil	Glyphosate	Rimsulfuron
Daminozide	Imazosulfuron	Spiroxamine
Dichlorprop-P	Imidacloprid	Sulcotrione
Difenoconazole	Iodosulfuron-methyl-sodium	Tebuconazole
Dimethachlor	Isoxaflutole	Thiacloprid

Dimethenamid-P	Lenacil	Thiamethoxam
Dimethoate (metabolite: omethoate)	Mancozeb	Thiram
Dinocap	Mesosulfuron	Tralkoxydim
Diquat (dibromide)	Mesotrione	Tri-allate
Ethofumesate	Metamitron	Tribenuron
Ethoprophos	Metconazole	Triticonazole

11.2.2. CAG level 2: Phenomenological / specific effects on the eye

Various types of effects on the eyes were identified as a basis for establishing CAGs at level 2. The cornea, the uvea/iris, the lens and the retina are primarily affected.

Based on these effects, four distinct CAGs at level 2 are proposed. More information is given in Appendix O.

11.2.2.1. CAG level 2a: Corneal opacity

The main effects reported in the cornea are opacity and keratitis (inflammation). These effects might be related, e.g. keratitis may lead to opacity. However, corneal opacity is not necessarily related to inflammation. The active substances identified as inducing corneal opacity are allocated to 'CAG level 2a: Corneal effects', whereas the substances inducing keratitis are allocated to CAG level 2d, see below.

For one substance, sulcotrione, keratopathy (a non-inflammatory disease of the cornea) was reported in the dog study, but not in the rat studies. This effect is also allocated to CAG level 2a.

The active substances allocated to CAG level 2a are listed in Table 11.2.

Table 11.2. CAG level 2a: Corneal opacity

Benfluralin	Isoxaflutole	Rimsulfuron
Chlorpropham	Mesotrione	Spiroxamine
Daminozide (metabolite: UDMH)	Metribuzin	Sulcotrione
Ethoprophos	Propineb (metabolite: PU)	Tri-allate

11.2.2.2. CAG level 2b: Cataract

The main effect reported in the lens is cataract. Any opacity of the lens and its capsule is clinically termed a cataract, a condition where the lens gradually changes from perfect transparency to translucency. A number of different terms have been used in the DARs to describe cataract, see Appendix Q for a list of these terms.

The active substances identified as inducing cataract are allocated to 'CAG level 2b: Cataract' and are listed in Table 11.3.

Table 11.3. CAG level 2b: Cataract

2,4-D	Dimethoate (metabolite: omethoate)	Mesotrione
2,4-DB	Dinocap	Metconazole
2-Phenylphenol	Diquat (dibromide)	Prosulfocarb
Acetamiprid	Famoxadone	Prothioconazole
Benfluralin	Fenhexamid	Rimsulfuron
Chlorothalonil (metabolite: SDS-3701)	Fenpropidin	Spiroxamine
Chlorpropham	Flazasulfuron	Sulcotrione
Chlorpyrifos	Flufenacet (formerly fluthiamide)	Tebuconazole
Cymoxanil	Glyphosate	Thiacloprid
Difenoconazole	Imazosulfuron	Thiamethoxam
Dimethachlor	Lenacil	Triticonazole
Dimethenamid-P	Mesosulfuron	

11.2.2.3. CAG level 2c: Retinal effects

A number of different effects have been reported for the retina including degeneration, atrophy, grey pigmentation / grey mottling / brown granularity of the tapetal fundus (membranous layer or region of the choroid / retina), degeneration of the optic fundus, hyperreflection, hyporeflection, tapetal lesions, dystrophy of the pigment epithelium, electroretinographic (ERG) abnormalities, cystic vacuolisation of the peripheral optic retina, and desquamation, see Appendix Q for a description of these effects. These effects might to some extent be interrelated. For the purpose of the CAG project, the abovementioned effects in the retina are allocated to a single CAG level 2, termed ‘CAG level 2c: Retinal effects’.

The active substances identified as inducing retinal effects and allocated to CAG level 2c are listed in Table 11.4.

Table 11.4. CAG level 2c: Retinal effects

2,4-D	Fluazinam	Oxasulfuron
2,4-DB	Flufenacet (formerly fluthiamide)	Penconazole
Acetamiprid	Fosthiazate	Propamocarb
Chlorothalonil, metabolite	Glufosinate	Spiroxamine
Chlorpyrifos	Imazosulfuron	Sulcotrione
Cymoxanil	Imidacloprid	Thiacloprid
Dichlorprop-P	Lenacil	Thiram
Dimethoate (metabolite: omethoate)	Mancozeb	Tralkoxydim
Dinocap	Metamitron	Tribenuron
Ethofumesate	Oxamyl	

11.2.2.4. CAG level 2d: Inflammation

A number of substances have been reported to induce inflammation around or in the eye, e.g. conjunctivitis (inflammation of the conjunctiva), keratitis (inflammation of the cornea), uveitis (inflammation of the uvea, which is formed of the iris, the ciliary body and the choroid), iritis (inflammation of the iris), and panophthalmitis (inflammation of the whole of the eye).

Other effects have also been reported, which are interpreted as being signs of inflammation, see Appendix Q for a list of these terms.

The active substances identified as inducing inflammation / signs of inflammation are allocated to 'CAG level 2d: Inflammation' and are listed in Table 11.5.

Table 11.5. CAG level 2d: Inflammation

2,4-D	Fenpropidin	Metconazole
2-Phenylphenol	Flazasulfuron	Sulcotrione
Acetamiprid	Imazosulfuron	Thiram
Chlorpyrifos	Iodisulfuron-methyl-sodium	Triticonazole
Difenoconazole	Isoxaflutole	
Dimethoate (metabolite: omethoate)	Mesotrione	

11.2.2.5. Effects not considered relevant for CAGs at level 2

Discolouration of an organ or tissue is generally not considered as being an adverse effect; however, for the eye, discolouration is accepted as an adverse effect. Discolouration of the eye or part of the eye has been reported for a number of the active substances identified as having an effect of the eyes. For some substances, the discolouration is observed in a particular part of the eye and thus, covered by the respective CAGs at level 2. For other substances, the discolouration is due to various effects not already included in a CAG at level 2, e.g. dark red discolouration due to haemorrhage. Although discolouration of the eye is regarded as an adverse effect, this effect is not considered as being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for effects on the eye.

Chromodacryorrhea, i.e. rats shedding red tears, is not caused by haemorrhage, but by the secretion of a red pigment, porphyrin, from a gland behind the eye (the Harderian gland). This effect is not considered as being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for effects on the eye.

Other effects on the eye have been noted for single substances, e.g. vacuolisation of the ciliary body epithelium, vacuolation and superficial exfoliation of epithelial cell (loosing layers of tissue), epithelial thickening, necrosis, subepithelial fibroblastic reaction and vascularisation of stroma (supporting tissue), fundal haemorrhage, miosis (contraction of the pupil), blepharospasms (sudden contraction of the eyelid), photophobia (the eyes become sensitive to light), and sclerosis (hardening of tissue). These effects are considered either as being non-

adverse effects and therefore, not relevant for CAGs at level 2, or as not being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for effects on the eye.

11.2.3. CAG level 3: Mode of action

For one type of the phenomenological / specific effects on the eye described under CAG level 2, a mode of action has been proposed. For the remaining substances, inadequate or no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

11.2.3.1. CAG level 3a1: Increased systemic tyrosine concentration

Increased systemic tyrosine concentration has been shown in a study of sulcotrione in rats to be accompanied by corneal lesions and keratitis (data summarised in the sulcotrione DAR July 2006). Isoxaflutole and mesotrione also increase the systemic tyrosine concentration in rats and, as for sulcotrione, this is accompanied by corneal lesions and keratitis (data summarised in the isoxaflutole March 1998 and mesotrione DAR December 1999, respectively).

The active substances inducing corneal opacity and keratitis (CAG level 2a) due to an increased systemic tyrosine concentration are allocated to CAG level 3a1 and are listed in Table 11.6.

Table 11.6. CAG level 3a1: Increased systemic tyrosine concentration

Isoxaflutole	Mesotrione	Sulcotrione

11.2.4. CAG level 4: Mechanism of action

For the active substances allocated to CAG level 3a1, a mechanism of action has been proposed. For the remaining substances, inadequate or no information regarding mechanisms of action has been found and consequently, these substances cannot be allocated to a CAG level 4.

11.2.4.1. CAG level 4a1a: HPPD inhibition

The mode of action for the two active substances of the triketone family included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009), mesotrione and sulcotrione, in both plants and animals is inhibition of the 4-hydroxyphenyl pyruvic acid dioxygenase (HPPD) enzyme, a key enzyme in the tyrosine catabolic pathway. Administration of an HPPD inhibitor results in increased systemic tyrosine concentrations, likely due to a competition of tyrosine and the accumulating substrate of HPPD (4-hydroxyphenyl pyruvate, 4-HPP) for the tyrosine transaminase enzyme (TAT) which can convert 4-HPP to 4-hydroxybenzaldehyde or re-convert it to tyrosine (Sulcotrione DAR July 2006, SCP 2002).

Data are available indicating that the main metabolite (2-cyano-3-cyclopropyl-1-(2-methylsulfonyl-4-trifluoromethylphenyl)propan-1,3-dione) of the active substance, isoxaflutole (the only isoxasole included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009)) is an HPPD inhibitor; isoxaflutole did not inhibit the enzyme (data summarised in the isoxaflutole DAR March 1998).

The active substances where corneal opacity and keratitis (CAG level 2a) are a result of increased systemic tyrosine concentration (CAG level 3a1) due to inhibition of HPPD are allocated to CAG level 4a1a and are listed in Table 11.7.

Table 11.7. CAG level 4a1a: HPPD inhibition

Isoxaflutole, main metabolite	Mesotrione	Sulcotrione

11.3. Discussion of CAGs for the eyes

Fifty-seven active substances were identified to have effects on the eye and were allocated to CAG level 1. Four distinct CAGs at level 2 have been proposed. Information on mode / mechanism of action is available for only three the active substances. The information is summarised in Appendix P.

11.3.1. Ad CAG level 4a1a: HPPD inhibition

As mentioned in section 1.2.4.1, administration of an HPPD inhibitor results in increased systemic tyrosine concentrations, likely due to a competition of tyrosine and the accumulating substrate of HPPD (4-hydroxyphenyl pyruvate, 4-HPP) for the tyrosine transaminase enzyme (TAT), which can convert 4-HPP to 4-hydroxybenzaldehyde or re-convert it to tyrosine. Alternative but normally inactive tyrosine catabolic pathways involve transformation of 4-HPP to 4-hydroxyphenyl lactic acid (4-HPLA), or of tyrosine itself to either N-acetyl tyrosine or 4-hydroxyphenyl acetic acid (4-HPAA). It is known that different species vary in their ability to metabolise tyrosine via other pathways and therefore show differing responses to administration of HPPD inhibitors. From the data available so far it has been concluded that rats (particularly male rats) are much more sensitive to the consequences of the enzyme inhibition on tyrosine so that the threshold tyrosine concentration for ocular effects can be reached in the rat after administration of far less HPPD inhibitor than would be needed in the mouse, monkey, or human. (Sulcotrione DAR, SCP 2002).

The Scientific Committee on Plants (SCP 2002) has summarised a number of volunteer studies of mesotrione and concluded that a tyrosine concentration threshold exists for the development of ocular lesions after HPPD inhibition, and further that in humans even complete inhibition of HPPD activity does not produce tyrosine concentrations greater than this threshold.

The corneal lesions seen with administration of HPPD inhibitors in rats have been accepted as a result of increased blood tyrosine or tyrosine metabolite concentrations and not relevant to humans (Sulcotrione DAR).

Thus, corneal opacities and keratitis resulting from administration of the two active substances of the triketone family included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009), mesotrione and sulcotrione, as well as isoxaflutole (the only isoxasole included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009)), to rats are not relevant for human risk assessment. Consequently, CAG level 4a1a and 3a1, as well as 2a and 2d for these three substances are not recommended for CRA for effects on the eye observed in rats. However, these CAGs are recommended for CRA for effects on the eye observed in mouse (mesotrione) and dog (mesotrione and sulcotrione).

11.3.2. Ad CAG level 2b: Cataract

The lens epithelium and the differentiating fibre cells in the lens bow are crucial for maintaining the state of hydration of the lens which is strongly dependent on the energy (ATP) content of the cells and on the activities of ion and water channels. The normal lens is in a "dehydrated" state. This strongly depends on the proper functioning of the ion and water pumps which derive their energy from the ATP delivered by lens epithelium and superficial lens fibres. The lens osmolarity is maintained by cations (Na^+ and K^+) and anions (Cl^-, bicarbonate, sulphate, ascorbate, glutathione, acidic groups of lens proteins and glycoproteins). Some of the markers used in various studies on cataractous ion changes in the lens include ATP-content, glutathione peroxidase and reductase activities, and ascorbate content. (SCP 2001).

Lenticular opacities can be caused by different mechanisms such as (SCP 2001, JMPR 2003):

- Changes in osmotic pressure caused by accumulation of active osmolytes, leading to an increased water content and lens weight which change the refractive properties and clarity of the lens (e.g. diabetic cataract).
- Post-translational denaturation of lens proteins, especially the crystallins, which are critical for maintaining lens clarity and refractive index (e.g. nuclear and cortical agerelated cataracts).
- Impairment of the normal differentiation process, including synthesis of crystallins, which is strongly dependent on unrestricted energy and nutrient supply. Dose-dependent reductions in lenticular ATP content correlate with a dose-dependent decrease in lenticular crystallin content. This effect seems not to be species specific (e.g. X-ray and steroid cataracts).

Nevertheless, the vast majority of chemicals induce cataracts with unknown mechanisms (SCP 2001).

It has been noted in some DARs that cataracts are frequent, age-induced manifestations with a variable incidence and occurring in the treated groups as well as in the control group and therefore, considered not likely to be a substance-induced effect. However, cataracts in long-term studies were considered in other DARs to be treatment-related. As no general conclusion

could be taken regarding the toxicological significance of cataracts in long-term studies based on the available data, active substances inducing cataracts in such studies have been allocated to CAG level 2b unless it was clear from the DAR that the incidences in treated groups were similar to that in the control group. Consequently, CAG level 2b is recommended for CRA for effects on the eye.

The active substances for which some information on mode / mechanism of action is available are addressed below.

11.3.2.1. Famoxadone

According to the famoxadone DAR, the precise mechanism of action of famoxadone (the only oxazole included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009)), in inducing cataracts in the dog is not known. However, mechanistic information indicates that it may involve some combination of effects on the metabolic state of the lens and/or the ion channels in the lens epithelium.

According to the famoxadone DAR, the known biochemical mechanism of action of famoxadone is inhibition of the mitochondrial respiratory chain at Complex III (ubiquinol:cytochrome c oxidoreductase) (HLO-1997-00761), resulting in decreased production of ATP by the cell. ATP is essential for maintenance of cellular metabolism and the function of ATP-dependent cellular enzymes, in particular the Na-K-ATPase which is critical to maintaining cellular hydration. Therefore, reduction in cellular ATP could result in an increase in lens hydration and subsequent cataract formation. Furthermore, ATP is required to maintain the reducing power of the cell, thus a decrease in ATP would increase susceptibility to oxidative stress, also implicated in cataract formation. A second, less well-characterised effect of famoxadone may be alterations in ion channel activity. Imbalances in cellular ion homeostasis, leading to increased cellular hydration, and alterations in calcium homeostasis, leading to protease activation, are both proposed mechanisms of cataract formation. These ion balances may be altered by famoxadone indirectly, through decreases in cellular energy (ATP) content. There is also limited evidence for a direct action of famoxadone on ion channels. *In vitro* exposure of cultured embryonic cockroach neurones to famoxadone produced inhibition of voltage-dependent sodium and calcium currents. Although direct ion channel effects have not been demonstrated *in vivo*, the *in vitro* data suggest ion channel imbalances may be another possible explanation for the toxicity of famoxadone in the lens.

According to the famoxadone DAR, the dog has been demonstrated to be relatively deficient (compared to primates) in numerous oxidative stress protective mechanisms. These deficiencies may underlie the observed increased sensitivity of the dog to lens toxicity, following exposure to famoxadone. There is strong support in the literature that the dog is not a good predictive model for assessing human risk of lens toxicity. Toxicity studies with famoxadone have demonstrated the lens toxicity to be specific to the dog and that a non-human primate species (cynomolgus monkey) is not susceptible to this toxicity when exposed to very high doses (1g/kg bw/day) for up to a year. Thus, the lens toxicity observed in dogs exposed to famoxadone should not be considered a relevant endpoint in assessing human risk from exposure to famoxadone. The ADI (0.088 mg/kg bw) was therefore based on the more sensitive endpoints excluding eye effects (haemolytic anaemia) in the 1-year dog study.

The Scientific Committee on Plants (SCP 2001) has summarised the experimental data for the active substance, famoxadone as follows: *"Famoxadone has no ocular irritancy potential. Ocular effects are observed in short and long term dog studies and in a two year rat study. The known biochemical mechanism of famoxadone is inhibition of the mitochondria respiratory chain at complex III resulting in decreased production of ATP by the cell (SCP/FAMOX/011). In the dog study in which high dosages of famoxadone were given an increase in serum K+ was found, indicating an effect on ion homeostasis at toxic doses. Metabolic studies conducted with famoxadone in rats and dogs demonstrated a longer half-life for radioactivity (parent and/or metabolite) in the dog, but no major qualitative differences in metabolic profile were found and the quantitative differences were only small (monograph, volume 3, annex B)."* Regarding the species specificity of the cataractogenic effect and the relevance to humans, the SCP came to another conclusion than that expressed in the famoxadone DAR: *"Experimental data show that famoxadone has a clear cataractogenic effect in Beagle dog. The precise mechanism of action of famoxadone is unknown. Results from studies carried out with other species (rats, mice and monkeys) do not allow a definite conclusion on the absence of cataractogenic effect in these species. Many compounds have been shown to be cataractogenic in either rats or Beagle dogs but very few compounds are cataractogenic in both species. The extrapolation of animal data to human is therefore difficult. The Committee is of the opinion that the eye effect of famoxadone in dogs is to be considered relevant for humans. This opinion could be revised with a more complete understanding of the mechanism of action of famoxadone on the eyes in the various species."*

JMPR (JMPR 2003) has summarised the experimental data for the active substance, famoxadone as follows: *"Exposure to famoxadone resulted in lens opacities and cataracts in outbred beagle dogs. These lesions (generally bilateral in occurrence) were observed after exposure to low doses of famoxadone in 90-day and 1-year studies. Exposures of similar and longer durations did not produce cataracts in mice or cynomolgus monkeys, despite exposure at much higher doses. Microscopically, however, cataracts were identified in the 18-month study in mice treated orally and in the 24-month study in rats treated orally. In mice there was clearly no dose-response relationship and in rats the excess incidence among males occurred at 40 and 400 mg/kg, but not at 200 mg/kg."* The available mechanistic data on famoxadone are summarised and discussed in detail in the monograph (JMPR 2003). JMPR recognised that the mechanism by which the lenticular effects in the dog are induced is not understood. JMPR concluded that the critical effect of famoxadone for the ADI was the occurrence of cataracts in dogs and the ADI (0-0.006 mg/kg bw) was established based on the NOAEL in the 1-year study in dogs. JMPR noted that some of these cataracts developed late in the study, indicating that progression might have been possible, had a long-term study been conducted.

According to the US-EPA, the biochemical mechanism of action of famoxadone is inhibition of the fungal mitochondrial respiratory chain at Complex III, resulting in a decreased production of ATP by the fungal cell (US-EPA 2003). The dose and endpoint for establishing the chronic reference dose (cRfD, 0.0014 mg/kg bw/day) was based on a LOAEL for treatment-related microscopic lens lesions (cataracts) in eyes of female dogs in the 13-week study as a NOAEL could not be determined. This view is also expressed in a recent rule (US-EPA 2009).

No further data have been found and therefore, allocation of famoxadone to a CAG level 3 or 4 may await further mechanistic data.

11.3.2.2. Fenpropidin, metconazole and triticonazole

According to the fenprodidin DAR, it has been shown that the cholesterol content and synthesis are important for the structural and functional integrity of the fiber cell plasma membrane in the lens and that a disturbance in cholesterol synthesis and/or the accumulation of cholesterol-oxides or -precursors can lead to cataract. Lower cholesterol levels in blood were reported in almost all subchronic studies with rats and dogs at higher dose levels of fenpropidin (chemical class: Unclassified). This may indicate an impairment of cholesterol synthesis by fenpropidin in analogy to its mode of action in fungi, where it impairs ergosterol synthesis by inhibiting Δ^{14}-reductase and Δ^{8}-Δ^{7}-isomerase. Both enzymes play a role for late steps of cholesterol synthesis in mammals. Therefore the observed cataracts in several animals after prolonged treatment with fenpropidin at high dose levels might have been mediated by an impairment of cholesterol synthesis in the eye.

For metconazole (a triazole), it is stated in the DAR that the cataractogenic potential of metconazole was related to the interference with steroid biosynthesis of the substance; however, no further details are presented in the DAR. It is noted that decreased plasma cholesterol concentrations have been reported in numerous studies.

For triticonazole (a triazole), it was stated by the study author (quoted in the DAT) that the cause of the lenticular degeneration in the dog is not clearly known, but such effects were observed with some hypocholesterolaemic agents, and the administration of triticonazole was clearly associated with lowered plasma cholesterol concentrations.

Other triazoles identified as inducing cataract in the dog are difenoconazole and tebuconazole, but exposure to these two triazoles did apparently not result in decreased plasma cholesterol concentrations. For penconazole, retinal effects but not cataract were reported. For the remaining triazoles (amitrole, epoxiconazole, flusilazole, propiconazole, tetraconazole and triadimenol) included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009), no effects on the eye were reported in the DARs.

No further data regarding a relation between lowered plasma cholesterol concentrations and cataract have been found and therefore, allocation of fenpropidin, metconazole and triticonazole to a CAG level 3 or 4 may await further mechanistic data.

11.3.3. Ad CAG level 2c: Retinal effects

The most common retinal effect reported is retinal degeneration / atrophy and most often in long-term studies. It has been discussed in some DARs whether this effect is an age-related phenomenon; however, no clear conclusion has been presented. Consequently, CAG level 2c is recommended for CRA for effects on the eye.

The active substances (flufenacet, dinocap, fluazinam, propamocarb) for which some information on mode / mechanism of action is available are addressed below.

11.3.3.1. Flufenacet

According to the flufenacet DAR, the toxicological profile suggests a sensitivity in the dog relative to thiadione excretion. The preponderance of scientific mode of action data support the contention that limitations in glutathione interdependent pathways and antioxidant stress, resulted in metabolic lesions or abiotrophism in the eye, brain and kidney of these dogs. No further data have been found and therefore, allocation of flufenacet to a CAG level 3 or 4 may await further mechanistic data.

11.3.3.2. Dinocap, fluazinam and propamocarb

For three active substances (dinocap, fluazinam and propamocarb), some of the retinal effects observed in dogs are related to the tapetal fundus / tapetal lucidum. It has been discussed in the propamocarb DAR) whether these effects are relevant to humans as no tapetum is present in humans, see below.

For propamocarb, degeneration of the optic fundus (retina) and hyporeflectivity were reported in the 90-day and 1-year dog studies. Loss of colour and reflectability of the tapetum lucidum of the ocular fundus was reported in the 2-year dog study. According to the DAR, hyporeflectibility of the fundus is ascribed to changes in the tapetum lucidum. Histopathological investigations (including electron microscopy) of these lesions indicated that the effects were due to degeneration of the cell-specific paraplasmatic inclusions (rodlets) and degenerative cytoplasmic changes in the tapetal cells. The cell organelles, however, were largely intact. No necrotic cells, macrophages or other cell infiltrates were demonstrated. The ocular tissues adjacent to the tapetum (retina and choroid) were ultrastructurally normal. According to the DAR, these lesions could be due to a deficiency of zinc produced by chelating properties of propamocarb and the effects were considered to be species specific as such lesions were not seen in animals lacking a tapetum lucidum. It is highly likely therefore, according to the DAR, that the absence of a tapetum in humans excludes the possibility of this ocular lesion occurring in man due to exposure to propamocarb.

For dinocap and fluazinam, effects on the tapetum lucidum were reported in the dog studies.

No further data have been found and therefore, allocation of dinocap, fluazinam and propamocarb to a CAG level 3 or 4, as well as a conclusion regarding the relevance of the findings in dogs for humans, may await further mechanistic data. Consequently, CAG level 2c is recommended for CRA for effects on the eye for these three active substances.

11.3.4. Chemical classes as basis for CAGs for the eye

Active substances belonging to the same chemical class may have similar toxicological effects. Information in the DARs on effects on the eye is summarised below for evaluation of similarity of toxicological effects within the relevant chemical classes, i.e. the chemical classes containing more than one active substance.

11.3.4.1. Aryloxyalkanoic acids

Three aryloxyalkanoic acids were identified to affect the eye: 2,4-D (cataract, retinal effects, inflammation), 2,4-DB (cataract, retinal effects) and dichlorprop-P (retinal effects). For the remaining aryloxyalkanoic acids (MCPA, MCPB, mecoprop, mecoprop-P), no effects on the

eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that aryloxyalkanoic acids in general have similar effects on the eye although it is acknowledged that similar effects have been reported for the three aryloxyalkanoic acids affecting the eye.

11.3.4.2. Carbamates

Three carbamates were identified to affect the eye: Chlorpropham (corneal opacity, cataract), oxamyl (retinal effects) and propamocarb (retinal effects). For the remaining carbamates (benthiavalicarb, iprovalicarb, methiocarb, methomyl, pirimicarb), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that carbamates in general have similar effects on the eye although it is acknowledged that similar effects have been reported for some of the carbamates.

11.3.4.3. Chloroacetamides

Two chloroacetamides were identified to affect the eye: Dimethachlor (cataract) and dimethenamid-P (cataract). For the remaining chloroacetamides (metazachlor, pethoxamid, S-metolachlor), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that chloroacetamides in general have similar effects on the eye although it is acknowledged that cataract has been reported for the two chloroacetamides affecting the eye.

11.3.4.4. Cyclohexadione oximes

One cyclohexadione oxime was identified to affect the eye: Tralkoxydim: (retinal effects). For the other cyclohexadione oxime (tepraloxydim), no effects on the eye were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that cyclohexadione oximes have similar effects on the eye.

11.3.4.5. Dithiocarbamates

Two dithiocarbamates and one metabolite of a dithiocarbamate were identified to affect the eye: Mancozeb (retinal effects), thiram (retinal effects, inflammation) and the propineb metabolite propylenurea (PU) (corneal clouding). For the remaining dithiocarbamates (maneb, metiram, propineb), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that dithiocarbamates in general have similar effects on the eye although it is acknowledged that retinal effects have been reported for the two dithiocarbamates affecting the eye.

11.3.4.6. Morpholines

One morpholine was identified to affect the eye: Spiroxamine (corneal opacity, cataract, retinal effects). For the other morpholines (demethomorph, fenpropimorph, dodemorph), no effects on the eye were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that morpholines have similar effects on the eye.

11.3.4.7. Neonicotinoids

Four neonicotinoids were identified to affect the eye: Acetamiprid (cataract, retinal effects, inflammation), imidacloprid (retinal effects), thiacloprid (cataract, retinal effects) and thiamethoxam (cataract). For the remaining neonicotinoid (clothianidin), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that neonicotinoids in general have similar effects on the eye although it is acknowledged that similar effects have been reported for some of the neonicotinoids.

11.3.4.8. Organophospates

Five organophospates were identified to affect the eye: Chlorpyrifos (cataract, retinal effects, inflammation), ethoprophos (corneal opacity), fosthiazate (retinal effects), glufosinate (retinal effects), phosmet (opacity – not further specified in the DAR, adhesion or coloboma). In addition, a metabolite (omethoate) of dimethoate was identified to affect the eye (corneal effects, cataract, retinal effects, inflammation). For the remaining organophosphates (chlorpyrifos-methyl, fenamiphos, fosetyl, pirimiphos-methyl), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that organophospates in general have similar effects on the eye although it is acknowledged that retinal atrophy has been reported for a few organophosphates.

It is noted that in the dimethoate DAR (July 2004) it is stated (in relation to the findings for omethoate) "*The finding most likely to be treatment-related is retinal degeneration, which has been reported previously for other organophosphate pesticides.*"

11.3.4.9. Sulfonylureas

Six sulfonylureas were identified to affect the eye: Flazasulfuron (cataract, inflammation), imazosulfuron (cataract, retinal effects, inflammation), iodosulfuron-methyl-sodium (inflammation), oxasulfuron (retinal effects), rimsulfuron (corneal opacity, cataract) and tribenuron (retinal effects).

For chlorsulfuron it is noted in the DAR (July 2007) "*With regard to ophthalmological examinations, these were only conducted in the 1-year dog study, as other subchronic studies were conducted prior to the availability of test guidelines requiring such examinations. An additional assessment was submitted (MacKenzie, 2004) evaluating histological data available for chlorsulfuron and histological and ophthalmological data of other structurally related sulfonylurea herbicides, as well as presenting ophthalmological versus histological detection of chemically induced ocular lesions. It was concluded that there is sufficient evidence to exclude the eye as a target organ for chlorsulfuron.*"

For mesosulfuron, the effects on the eye observed in the 2-year rat study (lens opacities) and in the 18-month mouse study (corneal and lens opacity) were considered as incidental changes in the DAR.

For the remaining sulfonylureas (amidosulfuron, azimsulfuron, bensulfuron, chlorsulfuron, ethoxysulfuron, flupyrsulfuron-methyl, metsulfuron-methyl, nicosulfuron, prosulfuron, sulfosulfuron, thifensulfuron-methyl, triasulfuron, triflusulfuron, tritosulfuron), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the

conclusion that sulfonylureas in general have similar effects on the eye although it is acknowledged that similar effects have been reported for some of the sulfonylureas.

11.3.4.10. Thiocarbamates

Two thiocarbamates were identified to affect the eye: Prosulfocarb (cataract) and tri-allate (corneal opacity). For the remaining thiocarbamate (molinate), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that thiocarbamates have similar effects on the eye.

11.3.4.11. Triazinones

Two triazinones were identified to affect the eye: Metamitron (retinal effects) and metribuzin (corneal opacity) No other triazinones are included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009). Therefore, the information in the DARs does not allow the conclusion that triazinones have similar effects on the eye.

11.3.4.12. Triazoles

Five triazoles were identified to affect the eye: Difenoconazole (cataract, inflammation), metconazole (cataract, inflammation), penconazole (retinal effects), tebuconazole (cataract) and triticonazole (cataract, inflammation). For propiconazole, effects on the eye (not further specified in the DAR) were observed in the 1-year dog study. For the remaining triazoles (amitrole, epoxiconazole, flusilazole, tetraconazole, triadimenol), no effects on the eye were reported in the DARs. Therefore, the information in the DARs does not allow the conclusion that that triazoles n general have similar effects on the eye although it is acknowledged that similar effects have been reported for four of the five triazoles affecting the eye.

11.3.4.13. Triketones

The two triketones (mesotrione and sulcotrione) have similar effects in the eye of rats with similar mode / mechanism of action, see section 11.3.1.

11.3.4.14. Conclusion: Chemical classes

Based on the analysis whether active substances belonging to the same chemical class may have similar effects on the eye it is concluded that active substances belonging to the same chemical class in general do not have similar effects on the eye. However, it is acknowledged that similar effects have been reported for some of the substances belonging to a particular chemical class.

11.4. Recommended CAGs for the eye

The following CAGs are recommended for CRA for effects on the eye:

- CAG level 4a1a: HPPD inhibition, see Table 11.7, mesotrione and sulcotrione – only if based on information from studies in dog and mouse.

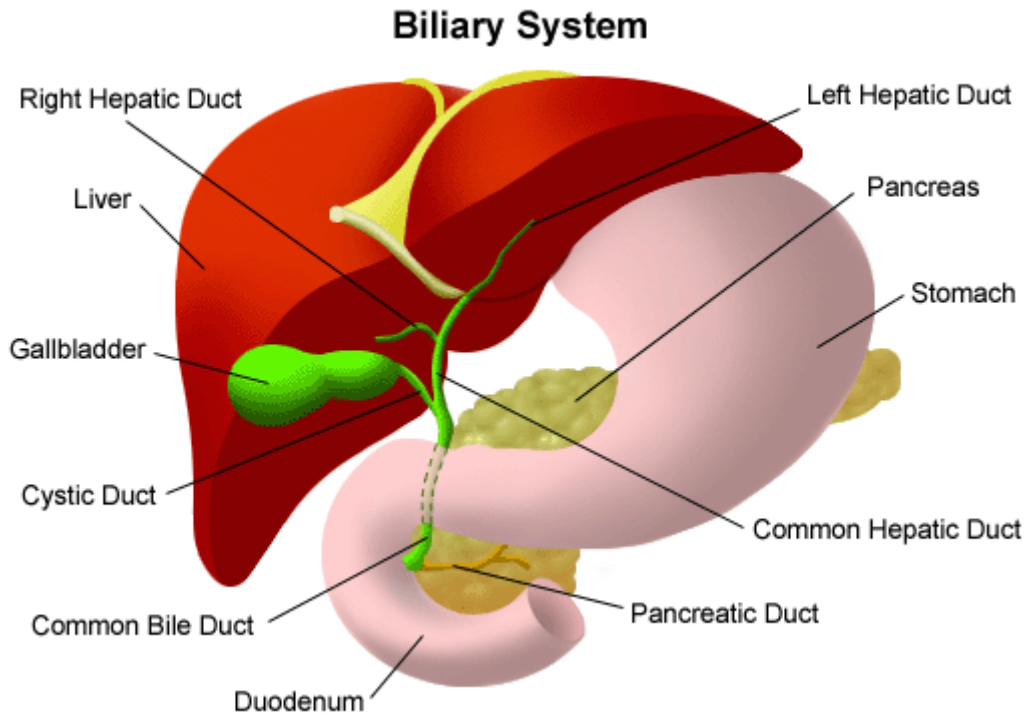
- CAG level 3a1: Increased systemic tyrosine concentration, see Table 11.6, mesotrione and sulcotrione – only if based on information from studies in dog and mouse.
- CAG level 2a: Corneal opacity, see Table 11.2, mesotrione and sulcotrione – only if based on information from studies in dog and mouse.
- CAG level 2b: Cataract, see Table 11.3.
- CAG level 2c: Retinal effects, see Table 11.4.
- CAG level 2d: Inflammation, see Table 11.5, mesotrione and sulcotrione – only if based on information from studies in dog and mouse.

12. Gallbladder

12.1. Introduction

The gallbladder is a saclike organ. Its primary function is to store and concentrate bile between meals. Bile is necessary for the digestion of lipids in the intestine. Bile is produced in the hepatocytes and flows through a network of different bile ducts into the common hepatic duct and the cystic duct into the gallbladder. The mucosa of the gallbladder wall readily absorbs water and electrolytes, leaving a high concentration of bile salts, bile pigments, and cholesterol. The gallbladder holds about 90 ml of bile. The bile is released into the duodenum within 30 minutes after eating. The bile release is controlled both by the nervous system and by the hormones cholecystokinin and motilin secreted by the duodenal mucosa in the presence of fat. It is bilirubin, which gives bile a yellowish green colour. (McCance and Huether 1998).

It should be noted that rats do not have a gallbladder. Consequently, no studies on gallbladder effects are available for rats.



From: http://www.cheboygansurgical.com/biliary-dyskinesia-80/

Figure 12.1. Anatomy of the gallbladder.

12.2. Establishment of CAGs for toxicity to the gallbladder

12.2.1. CAG level 1: Toxicity to the gallbladder

The active substances identified as having an effect on the gallbladder in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 12.1.

Table 12.1. CAG level 1: Toxicity to the gallbladder

Azimsulfuron	Cyhalofop-butyl	Nicosulfuron

12.2.2. CAG level 2: Phenomenological / specific effects on the gallbladder

One type of effects on the gallbladder was identified as a basis for establishing CAGs at level 2. Based on these effect, one distinct CAG at level 2 is proposed. More information is given in Appendix Q.

12.2.2.1. CAG level 2a: Hypertrophy / hyperplasia

Hypertrophy is an increased size of cells. Hyperplasia is an increased number of cells. Hyperplasia of the gallbladder may be a reaction to irritation of the gallbladder mucosa after xenobiotic exposure (Thoolen et al. 2010).

For the purpose of the CAG project, hypertrophy and hyperplasia are allocated to a single CAG level 2, termed 'CAG level 2a: Hypertrophy / hyperplasia'.

The active substances identified as inducing hypertrophy and/or hyperplasia of the gallbladder are allocated to CAG level 2a and are listed in Table 12.2.

Table 12.2. CAG level 2a: Hypertrophy / hyperplasia of the gall bladder

Azimsulfuron	Cyhalofop-butyl	Nicosulfuron

12.2.2.2. Effects not considered relevant for CAGs at level 2

For nicosulfuron dysplasia and calculi was noted. The calculi may be the cause of the epithelial hyperplasia and dysplasia. Dysplasia is delayed cell maturation and differentiation, and is often indicative of an early neoplastic process. Although hyperplasia and dysplasia is not the same, the dysplasia is considered covered by the CAG level 2a for hypertrophy/hyperplasia.

For the two active substances, azimsulfuron and cyhalofop-butyl, increased mucous secretion was noted. As the increased mucous secretion probably is a consequence of the same irritation that may be the cause of the epithelial hyperplasia, it is not considered relevant to create a CAG level 2 for increased mucous secretion.

Deposits of e.g. bile pigment is considered a non-adverse effect. Thus no CAG level 2 has been created for this effect.

12.2.2.3. Effects covered in the chapter about the liver

Lesions of the biliary epithelium such as hyperplasia and cysts, as well as cholestasis (a condition where bile cannot flow from the liver to the intestine) are addressed in the chapter on the liver (see chapter 17).

12.2.3. CAG level 3 / 4: Mode/mechanism of action

No information on modes/mechanisms of action have been found for any of the active substances identified as having an effect on the gallbladder.

12.3. Discussion of CAGs for the gallbladder

Three active substances were identified to have effects on the gallbladder and were allocated to CAG level 1. One distinct CAGs at level 2 has been proposed. No information on the modes/mechanisms of action is available. The information is summarised in Appendix R.

No information regarding the modes/mechanisms of action for the phenomenological effects in the gallbladder has been found. Therefore, the CAGs at level 2 could be considered for CRA.

12.4. Recommended CAGs for the gallbladder

The following CAG at level 2 is recommended for CRA for effects on the gallbladder:

- CAG level 2a: Hypertrophy / hyperplasia, see Table 12.2.

13. Gastrointestinal tract

13.1. Introduction

The gastrointestinal (GI) tract consists of the mouth, esophagus, stomach, small intestine, large intestine, rectum, and anus. It carries out the following digestive processes:

- Ingestion of food
- Propulsion of food and wastes from the mouth to the anus
- Secretion of mucus, water, and enzymes
- Mechanical and chemical digestion of food particles
- Absorption of digested food
- Elimination of waste products by defecation.

Histologically, the GI tract consists of four layers. From the inside out they are the mucosa, submucosa, muscularis, and serosa.

Disorders of the GI tract disrupt one or more of its functions. Structural and neural abnormalities can slow, obstruct, or accelerate the movement of chyme (partially digested food) at any level of the GI tract. Inflammatory and ulcerative conditions of the GI wall disrupt secretion, motility, and absorption.

13.2. Establishment of CAGs for toxicity to the gastrointestinal tract

Various types of effects on the gastrointestinal tract were identified including:

- Nausea

- Vomiting
- Diarrhoea
- Discolouration (white patches / zones, dark spots, reddening, red or black foci)
- Distension / dilatation (swelling / expansion)
- Hyperaemia (excess blood, here in the GI tract)
- Haemorrhage (bleeding)
- Irritation
- Ulcer
- Erosion
- Hyperplasia (increased number of cells)
- Acanthosis (diffuse epidermal hyperplasia)
- Hypertrophy (increased size of cells)
- Thickening
- Gastric stenosis (passage becomes narrow)
- Vacuolation
- Inflammation
- Gastritis (inflammation of the lining of the stomach)
- Atrophy (loss of tissue, totally or partially)
- Necrosis (death of cells and tissues)
- Tumours

Many of these effects in the GI tract are non-specific. Other effects are considered as being non-adverse.

Effects such as irritation, ulcer and erosion are often observed shortly following gastric intubation. Atrophy and/or necrosis may be a result of the irritating / corrosive property of a particular substance. Long-term oral exposure to irritating substances may lead to inflammation as well as hyperplasia / hypertrophy and even to tumours. Irritation and the consequente effects are generally related to the concentration of the substance rather than the dose.

Overall, the abovementioned effects are not considered relevant in terms of CRA for effects on the GI tract.

Therefore, the GI tract is not considered further for CAGs in this report.

13.3. Recommended CAGs for the gastrointestinal tract

No CAGs for toxicity to the gastrointestinal tract are recommended.

14. Haematological system

14.1. Introduction

The haematological system consists of the blood and bone marrow. Blood delivers oxygen and nutrients to all tissues, removes wastes, and transports gases, blood cells, immune cells, antibodies and hormones throughout the body.

Blood consist of various formed elements (cells and proteins) that circulate in the cardiovascular system suspended in plasma, which is approximately 90% water and 10% dissolved substances. All of these elements constitute the blood volume. Approximately 45-50% of the blood volume consists of the formed elements, and the remainder is the plasma.

The continuous movement of blood keeps the formed elements dispersed throughout the plasma, where they are able to carry out their chief functions: 1) delivery of substances needed for cellular metabolism in the tissues, 2) defence against invading micro-organisms and injury, and 3) acid-base balance.

The cellular elements of the blood are broadly classified as erythrocytes (red blood cells), leucocytes (white blood cells), and thrombocytes (platelets). Erythrocytes and thrombocytes function entirely within blood vessels; some leucocytes remain in the blood while others can enter tissues.

The erythrocytes are the most abundant cells of the blood and are responsible primarily for tissue oxygenation. Their cytoplasm consists of a solution containing protein (mostly haemoglobin, which carries the oxygen) and electrolytes. In healthy humans the total volume of circulating erythrocytes remains relatively constant. The feedback mechanism that maintains an optimal population of erythrocytes is mediated by erythropoietin. Erythropoietin (secreted by the kidney in response to tissue hypoxia) induces the production of erythrocytes (erythropoiesis), which takes place in the bone marrow. Erythrocytes are derived from precursor cells called erythroblasts (normal erythroblasts are called normoblasts, whereas abnormal ones are called megaloblasts). The erythroblasts loose the nucleus and the cell that remains is called a reticulocyte. The reticulocyte matures to an erythrocyte within 24-48 hours. Reticulocytes remain in the bone marrow approximately one day and then are released into the circulation and continue to mature in the blood. As the mature erythrocyte lacks the cytoplasmic organelles and cannot divide or carry out metabolic functions, it is replaced by a new erythrocyte generated in the bone marrow when it dies (life span approximately 120 days). The normal reticulocyte count is 1% of the total erythrocyte count and is therefore, a useful clinical parameter of erythropoietic activity and indicates whether new erythrocytes are being produced.

Leucocytes defend the body against organisms that cause infection and remove debris, including dead on injured host cells of all kinds. They act primarily in the tissues but are

transported in the circulation. Leucocytes are classified according to structure as either granulocytes or agranulocytes, and according to function as either phagocytes or immunocytes.

The granulocytes are so called because of the many lysosomal granules in their cytoplasm. They are all phagocytes and are classified as neutrophils (about 55% of the total leukocyte count), eosinophils (1-4% of the total leukocyte count), and basophils (<1% of total leukocyte count, also called mast cells).

The agranulocytes do not contain lysosomal granules in their cytoplasm. They are classified as macrophages, monocytes (immature macrophages), and lymphocytes (about 36% of the total leukocyte count).

All of the leucocytes arise from stem cells in the bone marrow. The granulocytes normally mature fully in the bone marrow and then are released into the bloodstream. The agranulocytes, however, are released into the bloodstream before they undergo their final phase of maturation.

Thrombocytes are not cells, but cytoplasmic fragments. They are formed in the bone marrow by fragmentation of very large cells known as megakaryocytes. Thrombocytes are essential for blood coagulation and control of bleeding. About 2/3 of the thrombocytes are in the circulating blood and the remaining 1/3 of the thrombocytes are in a reserve pool in the spleen.

In the short-term repeated dose toxicity studies (28- and 90-day), the haematological examinations should include the following parameters: Erythrocyte count (RBC count), haematocrit (Ht or packed cell volume (PCV)), haemoglobin concentration (Hgb), total and differential leucocyte count (WBC count), platelet count and a measure of blood clotting time/potential (generally the prothrombin time (PT) and activated partial thromboplastin time (APTT)). In the long-term repeated dose toxicity studies (chronic, chronic/carcinogenicity combined), the following red blood cell parameters should also be included: Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC). Other haematological parameters, such as e.g. Heinz bodies or other atypical erythrocyte morphology (anisocytosis, poikilocytosis), or methaemoglobin concentration may be included depending on known and/or suspected effects from a given substance. For a substance having an effect on the haematopoietic system, reticulocyte count and bone marrow cytology may also be included.

This section deals with the toxicity to the cellular elements of the blood, i.e. erythrocytes, leucocytes and thrombocytes. Toxicity to the bone marrow is addressed in section 8.

14.2. Establishment of CAGs for toxicity to haematological system

14.2.1. CAG level 1: Toxicity to the haematological system

The active substances identified as having an effect on the cellular elements of the blood in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 14.1.

Table 14.1. CAG level 1: Toxicity to the haematological system

1-Methyl-cyclopropene	Etoxazole	Molinate
2,4-D	Famoxadone	Oxadiazon
Acibenzolar-S-methyl (benzothiadiazole)	Fenamidone	Pethoxamid
Acibenzolar-S-methyl (benzothiadiazole)	Fenhexamid	Phenmedipham
Azimsulfuron	Fenoxaprop-P	Picloram
Benfluralin	Fenpropidin	Picolinafen
Bensulfuron	Fenpropimorph	Pirimicarb
Bentazone	Fipronil	Pirimicarb, metabolite R34836: desmethylpirimicarb
Benthiavalicarb	Flazasulfuron	Pirimicarb, metabolite R34885: desmethylformamidopirimicarb
Bifenazate	Florasulam	Prohexadione-calcium
Bifenox	Fluazinam	Propaquizafop
Bromoxynil	Fludioxonil	Prosulfocarb
Bromoxynil, metabolite 3,5-dibromo-4-hydroxybenzoic acid (DBHA)	Flufenacet (formerly fluthiamide)	Prosulfuron
Carfentrazone-ethyl	Flumioxazin	Pymetrozine
Chloridazon (aka pyrazone)	Fluopicolide	Pyraclostrobin
Chlorothalonil	Fluoxastrobin	Pyraflufen-ethyl
Chlorothalonil, metabolite SDS-3701: 4-hydroxy-2,5,6-trichloroisophtalonitrile	Forchlorfenuron	Pyrethrins
Chlorotoluron	Glufosinate	Pyriproxyfen
Chlorpropham	Imazosulfuron	Quinoclamine
Chlorsulfuron	Indoxacarb	Quinoxyfen
Chlorsulfuron, metabolite IN-A4097	Iodosulfuron-methyl-sodium	Quizalofop-P-ethyl
Cinidon ethyl	Ioxynil	Quizalofop-P-tefuryl
Clodinafop-prop	Iprodione	Spinosad
Clofentezine	Isoproturon	Sulcotrione
Clothianidin	Isoxaflutole	Tebuconazole
Cymoxanil	Linuron	Tebufenpyrad
Cyromazine	MCPA	Tepraloxydim
Daminozide	MCPB	Thiametoxam
Daminozide, metabolite UDMH (1,1-dimethylhydrazine)	Mancozeb	Thiophanate-methyl
Desmedipham	Mancozeb (metabolite ETU)	Tolclofos-methyl
Dicamba	Maneb	Tralkoxydim
Dichlorprop-P	Maneb (metabolite ETU)	Tri-allate
Difenoconazole	Mepiquat	Triasulfuron
Diflubenzuron	Metalaxyl-M	Triclopyr
Dimethachlor	Metamitron	Triclopyr, metabolite: 3,5,6-trichloro-2-pyridinol (TCP)
Dimoxystrobin	Metazachlor	Trifloxystrobin
Dinocap	Metconazole	Triflusulfuron
Diuron	Methomyl	Triticonazole
Epoxiconazole	Methoxyfenozide	Tritosulfuron
Ethoprophos	Metiram	Ziram
Ethoxysulfuron	Metribuzin	

Etofenprox	Milbemectin	

14.2.2. CAG level 2: Phenomenological / specific effects on the cellular elements of blood

Various types of effects on the cellular elements of the blood were identified as a basis for establishing CAGs at level 2. Based on these effects, three distinct CAGs at level 2 are proposed. More information is given in Appendix S.

14.2.2.1. CAG level 2a: Anaemia

Strictly speaking, anaemia is not a disease, but a term indicating a condition where the number of erythrocytes or the concentration of haemoglobin is less than normal, resulting in an insufficient supply of oxygen to the cells.

The following haematological parameters are all signs of anaemia and are, for the purpose of the CAG project, therefore allocated to a single CAG level 2, termed ‘CAG level 2a: Anaemia’ (see Appendix U for a list of the terms that are interpreted as anaemia / signs of anaemia):

- Decreased red blood cell (RBC) count
- Decreased haemoglobin concentration
- Decreased haematocrit (relates to the percentage of a given volume of blood occupied by red blood cells)
- Increased / decreased MCV (relates to the average size of the red blood cell)
- Increased / decreased MCH (relates to the amount of haemoglobin in a single red blood cell by weight)
- Increased / decreased MCHC (relates to the amount of haemoglobin in a single red blood cell by percentage)
- Increased / decreased reticulocyte count (reticulocytosis / reticulocytopenia)
- Increase in Heinz bodies
- Anisocytosis (the erythrocytes have variable and abnormal size)
- Poikilocytosis (the erythrocytes have various shapes)
- Increased methaemoglobin concentration

Anaemia is usually a result of 1) impaired erythrocyte production, 2) blood loss, 3) increased erythrocyte destruction, or 4) a combination of the three.

Anaemias are classified according to their aetiology or morphological basis. The most common classification of anaemias is based on cellular morphological structure. Descriptions of anaemias based on erythrocyte morphology refer to the size of the cells (terms end with ‘cytic’) and concentration of haemoglobin (terms end with ‘chromic’). The changes in MCV,

MCH and MCHC are used for classification of anaemias and therefore, could be useful in considerations of mode of action. As an example, the microcytic-hypochromic anaemias are characterised by erythrocytes that are abnormally small (MCV decreased) and contain abnormally decreased amounts of haemoglobin (MCH/MCHC decreased).

The reticulocyte count is a useful clinical index of erythropoietic activity in the bone marrow and indicates whether new red blood cells are being produced in the bone marrow. Reticulocytosis indicates a normal function of the bone marrow whereas reticulocytopenia indicates that the bone marrow is damaged more or less.

The active substances identified as inducing one or more of the above-mentioned effects are allocated to CAG level 2a and are listed in Table 14.2.

Table 14.2. CAG level 2a: Anaemia

1-Methyl-cyclopropene	Etoxazole	Molinate
2,4-D	Famoxadone	Oxadiazon
Acibenzolar-S-methyl (benzothiadiazole)	Fenamidone	Pethoxamid
Acibenzolar-S-methyl (benzothiadiazole)	Fenhexamid	Phenmedipham
Azimsulfuron	Fenoxaprop-P	Picloram
Benfluralin	Fenpropidin	Picolinafen
Bensulfuron	Fenpropimorph	Pirimicarb
Bentazone	Fipronil	Pirimicarb, metabolite R34836: desmethylpirimicarb
Benthiavalicarb	Flazasulfuron	Pirimicarb, metabolite R34885: desmethylformamidopirimicarb
Bifenazate	Florasulam	Prohexadione-calcium
Bifenox	Fluazinam	Propaquizafop
Bromoxynil	Fludioxonil	Prosulfocarb
Bromoxynil, metabolite 3,5-dibromo-4-hydroxybenzoic acid (DBHA)	Flufenacet (formerly fluthiamide)	Prosulfuron
Carfentrazone-ethyl	Flumioxazin	Pymetrozine
Chloridazon (aka pyrazone)	Fluopicolide	Pyraclostrobin
Chlorothalonil	Fluoxastrobin	Pyraflufen-ethyl
Chlorothalonil, metabolite SDS-3701: 4-hydroxy-2,5,6-trichloroisophtalonitrile	Forchlorfenuron	Pyrethrins
Chlorotoluron	Glufosinate	Pyriproxyfen
Chlorpropham	Imazosulfuron	Quinoclamine
Chlorsulfuron	Indoxacarb	Quinoxyfen
Chlorsulfuron, metabolite IN-A4097	Iodosulfuron-methyl-sodium	Quizalofop-P-ethyl
Cinidon ethyl	Ioxynil	Quizalofop-P-tefuryl
Clodinafop-prop	Iprodione	Spinosad
Clofentezine	Isoproturon	Sulcotrione
Clothianidin	Isoxaflutole	Tebuconazole
Cymoxanil	Linuron	Tebufenpyrad
Cyromazine	MCPA	Tepraloxydim

Daminozide	MCPB	Thiametoxam
Daminozide, metabolite UDMH (1,1-dimethylhydrazine)	Mancozeb	Thiophanate-methyl
Desmedipham	Mancozeb (metabolite ETU)	Tolclofos-methyl
Dicamba	Maneb	Tralkoxydim
Dichlorprop-P	Maneb (metabolite ETU)	Tri-allate
Difenoconazole	Mepiquat	Triasulfuron
Diflubenzuron	Metalaxyl-M	Triclopyr
Dimethachlor	Metamitron	Triclopyr, metabolite: 3,5,6-trichloro-2-pyridinol (TCP)
Dimoxystrobin	Metazachlor	Trifloxystrobin
Dinocap	Metconazole	Triflusulfuron
Diuron	Methomyl	Triticonazole
Epoxiconazole	Methoxyfenozide	Tritosulfuron
Ethoprophos	Metiram	Ziram
Ethoxysulfuron	Metribuzin	
Etofenprox	Milbemectin	

14.2.2.2. CAG level 2b: Thrombocytosis

Thrombocytosis is a condition where the level of thrombocytes (platelets) is higher than normal.

The active substances identified as increasing the number of thrombocytes are allocated to CAG level 2b and are listed in Table 14.3.

Table 14.3. CAG level 2b: Thrombocytosis

Azimsulfuron	Indoxacarb	Prosulfocarb
Benfluralin	Isoxaflutole	Pyriproxyfen
Benthiavalicarb	Linuron	Quinoclamine
Bifenazate	Metalaxyl-M	Sulcotrione
Chlorpropham	Metazachlor	Tebuconazole
Clodinafop-prop	Methomyl	Tebufenpyrad
Clofentezine	Methoxyfenozide	Tepraloxydim
Desmedipham	Milbemectin	Thiophanate-methyl
Etoxazole	Molinate	Tralkoxydim
Fludioxonil	Pethoxamid	Tri-allate
Flufenacet (formerly fluthiamide)	Phenmedipham	Triasulfuron
Forchlorfenuron	Pirimicarb	Trifloxystrobin

14.2.2.3. CAG level 2c: Thrombocytopenia

Thrombocytopenia is a condition where the level of thrombocytes (platelets) is less than normal.

The active substances identified as decreasing the number of platelets are allocated to CAG level 2c and are listed in Table 14.4.

Table 14.4. CAG level 2c: Thrombocytopenia

2,4-D	Indoxacarb	Prosulfuron
Azimsulfuron	MCPA	Pymetrozine
Chlorpropham	MCPB	Pyriproxyfen
Clodinafop-prop	Metconazole	Tebufenpyrad
Clothianidin	Metribuzin	Triclopyr
Difenoconazole	Pethoxamid	Triticonazole

14.2.2.4. Effects not considered relevant for CAGs at level 2

Anaemia is usually a result of 1) impaired erythrocyte production, 2) blood loss, 3) increased erythrocyte destruction, or 4) a combination of the three. Anaemia caused by impaired erythrocyte production is secondary to a direct damage to the bone marrow and anaemia due to blood loss is an unspecific effect and therefore, not relevant for CAG level 2a and consequently, not relevant in terms of CRA for anaemia.

Leucocytosis is a condition where the level of leucocytes (white blood cells) is higher than normal and leucopenia is a condition where the level of leucocytes is less than normal. CAGs at level 2 could be considered for both leucocytosis and for leucepenia. Alterations in white blood cells (WBC) are evaluated by measuring the total and differential leucocyte count (WBC count) in the haematological examination. In the DARs alterations in WBC are described in general terms such as e.g. increased/decreased WBC as well as in more specific terms such as increase / decrease in the various types of WBCs (neutrophils, eosinophils, basophils, monocytes, lymphocytes). Many of the active substances included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009) show an alteration in the WBC count and/or in one or more of the various types of WBCs. Generally, the alterations are only slight in comparison with the control group, within the normal range, not dose-related and/or only observed at very high doses. For some substances there is a general consistency in the findings across species and studies, but for most substances the alterations are only noted in one species and/or in one sex and/or only in one or a few of the studies. Furthermore, the alterations are often not consistent in terms of the direction of the alteration, e.g. increased count in one sex but decreased in the other sex, increased count in one study but decreased count in another study, and/or increased count in one species but decreased count in (an)other species. Therefore, the alterations in WBC counts are often considered in the DARs not to be treatment-related. Moreover, the descriptions in the various DARs are very different regarding details and preciseness. Finally, the alterations in WBC counts are generally not due to a direct damage of the WBCs in the blood but secondary to effects and disorders in other target organs and tissues such as the lymphoid organs and tissues. In conclusion, alterations in WBC counts for the active substances included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009) are considered either as being non-adverse effects and therefore, not relevant for

CAGs at level 2, or as not being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for either leucocytosis or leucopenia.

Reticulocytopenia is observed in some forms of anaemias, e.g. pernicious anaemia (disease where an inability to absorb vitamin B_{12} prevents the production of red blood cells), folate deficiency anaemia and aplastic anaemia (anaemia caused by bone marrow failure). Of these types of anaemia, only aplastic anaemia is relevant for pesticidal active substances. Reticulocytopenia has been reported for one active substance (chlorothalonil) in a single 1-year study on dogs whereas this effect was not reported in the other 1-year study on dogs as well as in the studies on rodent species. Reticulocytopenia has also been reported for a metabolite of an active substance (acibenzolar-S-methyl), a metabolite that is cytotoxic to the bone marrow. As aplastic anaemia is secondary to a direct damage to the bone marrow, this sign of anaemia is not considered relevant for CAG level 2a and consequently, not relevant in terms of CRA for anaemia.

14.2.3. CAG level 3: Mode of action

For a few of the active substances affecting the red blood cells and allocated to CAG level 2a, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

14.2.3.1. CAG level 3a1: Haemolysis

Anaemia caused by increased erythrocyte destruction is a direct effect on the red blood cells. Haemolytic anaemia is one form of anaemia in which the red blood cells are destroyed and removed from the bloodstream earlier than normally. Haemolytic anaemia can be intravascular (red blood cells in the blood stream) as well as extravascular (red blood cells outside the blood vessels).

The reticulocyte count is usually increased in haemolytic anaemia and thus, reticulocytosis is a good indication whether anaemia could be considered as being due to haemolysis. Also increases in Heinz bodies and/or Howell-Jolly bodies are indications of haemolytic anaemia. However, reticulocytosis may also be solely due to a general bone marrow response in order to compensate for the anaemia.

For the purpose of the CAG project, active substances causing anaemia accompanied by reticulocytosis, Heinz bodies and/or Howell-Jolly bodies in one or more of the studies of the active substances and/or their metabolite(s) are only allocated to CAG level 3a1 provided that haemolytic anaemia is justified based on information in the DAR or in the open literature.

In haemolytic anaemia (intravascular), the concentration of methaemoglobin is generally increased. For the purpose of the CAG project, active substances causing methaemoglobinaemia are only allocated to CAG level 3a1 provided that haemolytic anaemia is justified based on information in the DAR or in the open literature.

The active substances allocated to CAG level 3a1 are listed in Table 14.5. It should be noted that the CAG level 3a1 only covers substances inducing intravascular haemolytic anaemia. The substances causing methaemoglobinaemia are marked with the abbreviation ‘MetHb’ in

the table. One active substance also caused sulfhaemoglobinaemia; this substance is marked with the abbreviation 'SulfHb' in the table.

Table 14.5. CAG level 3a1: Haemolysis

1-Methyl-cyclopropene	Famoxadone	Methomyl
Acibenzolar-S-methyl (benzothiadiazole)	Indoxacarb (MetHb)	Metribuzin
Benthiavalicarb	Isoproturon (MetHb)	Molinate
Bifenazate	Linuron (MetHb)	Picolinafen (MetHb)
Desmedipham (MetHb)	MCPA	Prosulfocarb
Diflubenzuron MetHb, SulfHb)	Metazachlor	Triasulfuron
Diuron		

14.2.3.2. CAG level 3a2: Oxidative stress

For one active substance, methomyl, information in the open literature suggests that the haemolytic anaemia is caused by oxidative stress as indicated by increasing lipid peroxidation and perturbations in various antioxidant enzymes (Mansour et al. 2001). Garg et al. (2008) have noted that change in the membrane lipid composition is the key factor for deformations in blood-cell shape (e.g. spherocytosis and poikilocytosis) in response to various adverse treatments. The other factor might be the formation of active loci on the red blood cell membrane as a result of interaction with free radicals, thereby resulting in the alteration of the shape of red blood cells. This is, according to the authors, consistent with the increase in lipid oxidation observed in their own study. It is not clear whether the haemolytic anaemia is intravascular or extravascular.

For another active substance, acibenzolar-S-methyl, information in the DAR indicates that the haemolytic anaemia is caused by oxidative stress via metabolic release of methanethiol (an indirect mechanism) with the consequence of increased extravascular erythrocyte degradation.

The active substances identified as inducing haemolytic anaemia by oxidative stress are allocated to CAG level 3a2 and are listed in Table 14.6.

Table 14.6. CAG level 3a2: Oxidative stress

Acibenzolar-S-methyl (benzothiadiazole)	Methomyl	

14.2.4. CAG level 4: Mechanism of action

For a few of the active substances affecting the red blood cells, a mechanism of action has been proposed. For the remaining substances, no information regarding mechanisms has been found and consequently, these substances cannot be allocated to a CAG level 4.

14.2.4.1. CAG level 4a1a: Reaction with sulfhydryl groups on the surface of erythrocytes

For one active substance, 1-methylcyclopropene, information in the DAR indicates that the haemolytic anaemia is caused by a reaction of the ethylene bond in 1-methylcyclopropene with sulfhydryl groups on the surface of erythrocytes. This substance is allocated to CAG level 4a1a and is listed in Table 14.7.

Table 14.7. CAG level 4a1a: Reaction with sulfhydryl groups on the surface of erythrocytes

1-Methyl-cyclopropene		

14.2.4.2. CAG level 4d1a: Immune reaction

For one active substance, azimsulfuron, information in the DAR indicates that the anaemia observed in dogs is due to azimsulfuron acting as a hapten to induce an immune reaction against the circulating blood cells, i.e. an immune mediated destruction of blood cells. This substance is allocated to CAG level 4d1a and is listed in Table 14.8.

Table 14.8. CAG level 4d1a: Immune reaction

Azimsulfuron		

14.3. Discussion of CAGs for the haematological system

One hundred and twentyfour active substances were identified to affect the cellular elements of the blood and were allocated to CAG level 1. Three distinct CAGs at level 2 have been proposed. Information on mode/mechanism of action is available for only a few of the active substances. The information is summarised in Appendix T.

Nineteen substances were identified as inducing haemolytic anaemia (CAG level 3a1, Table 14.5). The CAG level 3a1 is recommended for CRA. Six of the substances also induced methaemoglobinanemia. In the clinic, one of the parameters examined in order to determine whether an anaemia is of the haemolysis is intravascular is methaemoglobinanemia. However, it is not clear whether haemolytic anaemia always is accompanied by methaemoglobinanemia.

Therefore, the other active substances identified as inducing methaemoglobinanemia were not included in the CAG level 3a1.

In addition to the six substances identified to induce both haemolytic anaemia and methaemoglobinanemia, six other substances were identified as inducing methaemoglobinanemia: Beflubutamid, chlorpropham, clodinapob-prop, flufenacet, methoxyfenozide and phenmedipham. Whether these substances also induce haemolytic anaemia is not clear from neither the DAR nor the open literature. It has been considered whether a specific CAG for methaemoglobinanemia would be relevant. However, as mentioned, the available information is considered insufficient in order to conclude whether methaemoglobinanemia might be or might not be associated with haemolytic anaemia. Moreover, it is not clear whether methaemoglobinanemia is just a sign of anaemia in general and thus covered by CAG level 2a, or whether methaemoglobinanemia is a specific type of anaemia for which a specific CAG at level 3 would be relevant, i.e. it is not clear whether a CAG level 2 or a CAG level 3 would be the relevant one for methaemoglobinanemia. Therefore, a specific CAG for methaemoglobinanemia is not recommended for the time being.

Two substances were identified as inducing anaemia via oxidative stress (CAG level 3a2, Table 14.6). For one substance, methomyl, it is not clear whether the oxidative stress is related to intravascular or extravascular haemolytic anaemia. For the other substance, acibenzolar-S-methyl, the oxidative stress led to increased extravascular erythrocyte degradation. The CAG level 3a2 is therefore, not recommended for CRA for the time being.

For iodosulfuron-methyl-sodium, the haematotoxicity in dogs was interpreted in the DAR as an interaction of the test compound at high concentrations with cell maturation in the haematopoietic tissues, mainly the bone marrow. Thus, the anaemia could be considered as secondary to the effect in the bone marrow and therefore, not relevant for CAG level 2a and consequently, not relevant in terms of CRA for anaemia. However, in the bone marrow, iodosulfuron-methyl-sodium was reported to increase in myeloid to erythroid ratio and to induce hyperplasia, effects which have been interpreted as being secondary to anaemia and consequently, not relevant in terms of CRA for effects on the bone marrow. As no further information has been located, iodosulfuron-methyl-sodium has not been allocated to a CAG level 3.

For two substances, a mechanism of action has been proposed: 1-Methyl-cyclopropene (CAG level 4a1a, Table 14.7) and azimsulfuron (CAG level 4d1a, Table 14.8). However, as only one active substance is to each of these two CAGs at level 4, these two CAGs are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active substances reveals the mechanism of action forming the basis for the respective CAGs.

For carfentrazone-ethyl it is noted in the DAR "*It is believed to be an inhibitor of protoporphyrinogen oxidase and of heme synthesis in animals.*" As no further information has been located, carfentrazone-ethyl can not been allocated to a CAG level 3 or 4.

No information regarding the mode of action for the remaining active substances allocated to CAG level 2a has been found. Therefore, this CAG at level 2 could be considered for CRA for anaemia in general.

Regarding effects on the platelets, thrombocytosis (CAG level 2b) as well as thrombocytopenia (CAG level 2c), no information on mode/mechanism of action has been found except for benthiavalicarb where it has been indicated in the DAR that thrombocytosis may be linked to a chronic inflammatory response. Therefore, these CAGs at level 2 could be considered for CRA for effects on the platelets.

14.4. Recommended CAGs for the haematological system

The following CAGs are recommended for CRA for effects on the cellular elements in the blood, which is a part of the haematological system (the other part, the bone marrow, is addressed in chapter 8):

- CAG level 3a1: Haemolysis, see Table 14.5.
- CAG level 2a: Anaemia, see Table 14.2.
- CAG level 2b: Thrombocytosis, see Table 14.3.
- CAG level 2c: Thrombocytopenia, see Table 14.4.

The following CAGs are not recommended for CRA for the time being. However, these CAGs may become relevant in the future provided that new information on other active substances justifies the mode of action / phenomenological effects forming the basis for the respective CAGs:

- CAG level 3a2: Oxidative stress, see Table 14.6.
- CAG level 4a1a: Reaction with sulfhydryl groups on the surface of erythrocytes, see Table 14.7.
- CAG level 2b: Immune reaction, see Table 14.8.

15. Immune system

15.1. Introduction

The immune system is represented in most tissues, organs and peripheral sites and is therefore readily accessible for toxic substances regardless of the route of exposure. It is designed to distinguish between self and non-self to provide host integrity against invading pathogens and emerging tumour cells as well as to respond to tissue damage and external potential host-threatening stimuli. As the immune system works by means of recognition and response, it is important in toxicological risk assessment not only to consider whether chemical exposure may results in insufficient immune responses but also whether it may results in inappropriate immune responses in terms of disease risk.

Bone marrow, liver, thymus and Peyer’s patches represent primary sites of the immune system that is important for immune cell maturation. Specific secondary lymphoid sites such as the

spleen, lymph nodes and tonsils are important for antigen presentation and initiation of adaptive immune responses. Mucosal-associated lymphoid tissue is regional organisations of immune cell populations that are important in local immunity; examples are the bronchus-associated lymphoid tissue and gut-associated lymphoid tissue associated with respiratory and oral exposure, respectively.

The mammalian immune system is composed of two cooperating subdivisions: The innate immune system and the adaptive immune system. These can be separated by the rapidity and specificity of the response. The innate immune system comprises of anatomical, humoral and cellular barriers and provides a rapid immediate defence with a low degree of specificity. On the other hand, adaptive immune responses have a much slower onset, a high degree of specificity, recognise a much broader range of foreign substances and produce signals and components that increase the innate response. Furthermore, adaptive immunity has three unique features: Diversity, immunological memory and self-nonself recognition; and can be divided into humoral immunity (response mediated by B cell-secreted antibodies) and cell-mediated immunity (response mediated by antigen-specific T cells in collaboration with various non-specific immune cells). Contrary to innate immunity which is found in all classes of plant and animal life, adaptive immunity is only found in vertebrates.

One way to evaluate immune system status is to examine the host response to challenge with an infectious agent or immunisation with a foreign antigen. Thus, it is fundamental to evaluate functional immune responses to antigenic challenge to detect chemically induced adverse effects (such as immune system impairment or dysfunction) influencing health risk. In general, evaluation of structural alterations of the resting/unchallenged immune system (e.g. immunopathological examination) is considered less sensitive to detect immunotoxic effects compared to measurement of the functional immune response to antigenic challenge. Finally, it is important to notice that dose sensitivity may be greater in early life than in adulthood as well as early-life immune insults may result in immune dysfunction affecting later-life host integrity and diseases.

15.2. Establishment of CAGs for toxicity to the immune system

Most of the immune system-related changes described in DARs are based on studies not conducted according to test guideline toxicity or immunotoxicity studies. In addition, most of the studies lack information about study design and examined parameters which complicate assessment of NOAELs and LOAELs. Thus, the effects on the immune system reported most probably reflect a state of physiological stress (in general most studies noted increased adrenal weights). A few studies demonstrated an immunomodulating effect but were not able to identify or clarify the underlying mechanism(s).

Effects on the primary sites of the immune system, i.e. bone marrow, liver and thymus, and the lymph nodes are described in the sections of these organs, respectively.

Therefore, the immune system is not considered further for CAGs in this report.

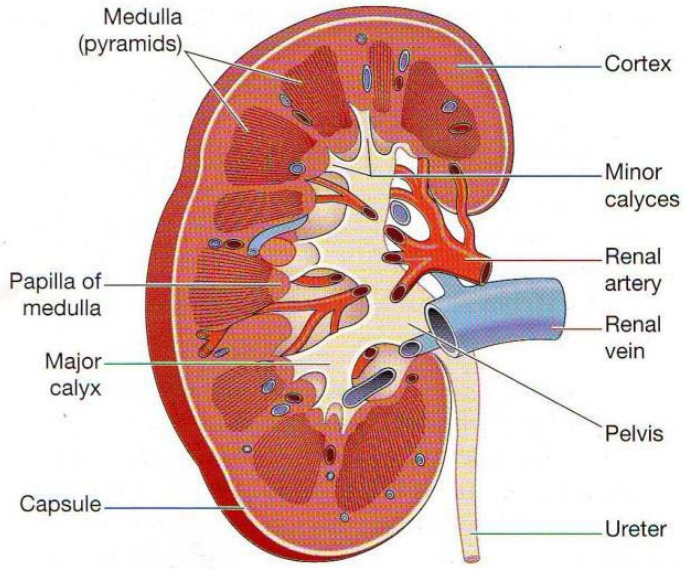
15.3. Recommended CAGs for the immune system

No CAGs for toxicity to the immune system are recommended.

16. Kidney

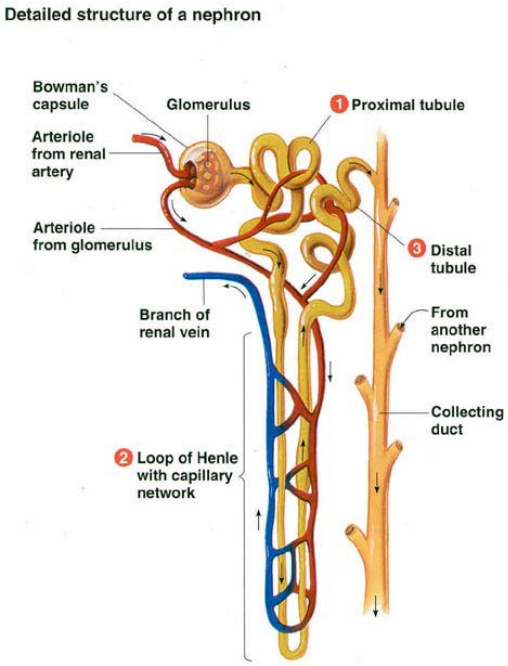
16.1. Introduction

The kidneys are paired organs. Each kidney has a bean-shaped structure and consists of a capsule, the outer renal cortex and the inner renal medulla (see Figure 16.1.) The medulla contains a number of cone-shaped renal pyramids. The tip of each pyramid is called the papilla. The functional unit of the kidney is the nephron (see Figure 16.2.). More than a million nephrons are located in each kidney in the cortex and medulla. The nephron consists of the glomerulus, which is a tuft of capillaries. The main function of the glomerulus is to filter the blood. The glomerulus is located in Bowman´s capsule. From Bowman´s capsule the filtered blood is lead into the tubules, which are subdivided into various segments such as the proximal tubule, loop of Henle and the distal tubule. The function of the tubules is to regulate the filtrate to maintain body fluid volume, electrolyte composition, and pH within narrow limits. The urine formed by the nephrons in the kidney flow from the distal tubules and collecting ducts through the renal papilla, and into the minor and major calyces, and is collected in the renal pelvis. From the renal pelvis, urine is funnelled into the ureters and on to the bladder. In addition to maintaining a stable internal environment, the kidneys also secrete hormones for regulation of blood pressure, erythrocyte production, and calcium metabolism. (McCance and Huether 1998).



A longitudinal section of the right kidney.

From http://faculty.ksu.edu.sa/75719/Pictures%20Library/



From http://osmoregulation-

Urinary%20system/The%20kidney,%20LS.jpg

Figure 16.1. Anatomy of the kidney.

apbio3.wikispaces.com/Kidney

Figure 16.1. Structure of a nephron.

The urinary passages, the renal pelvis, the ureters, the bladder and the urethra are histological similar to each other. They have a mucosal layer lined with transitional epithelium. Underlying the mucosal layer are muscle layers covered by a serous membrane or connective tissue. (Verlander 1998). Therefore the pathogenesis for effects in the renal pelvis and ureters may be the same as for effects in the urinary bladder and urethra (Cohen 1998). Effects in the urinary bladder and urethra have been included in the chapter about the urinary bladder.

Any chemical in the blood will be delivered to the kidney in relatively high amounts as the kidneys receive about 25 percent of the resting cardiac output. In addition potential toxicants may be concentrated in the tubular fluid, thereby driving passive diffusion of toxicants into the tubular cells. Therefore, a non-toxic concentration of a chemical in the blood may reach toxic concentrations in the kidney. Progressive concentration of toxicants along the nephron may result in precipitation and ultimately calculi, which may obstruct the renal flow of urine. Many nephrotoxicants have their primary effects on discrete regions of the nephron. The proximal tubule is the most common site of toxicant-induced renal injury. Part of the reason is that the proximal tubules compared to the distal tubules have a much more leaky epithelium favouring the flux of compounds into the proximal tubular cells. Furthermore, active transport of many molecules into the tubular cells is greater in the proximal tubules than in other segments of the tubules. (Klaassen 1996).

16.2. Establishment of CAGs for toxicity to the kidney

16.2.1. CAG level 1: Toxicity to the kidney

The active substances identified as having an effect on the kidney in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 16.1.

Table 16.1. CAG level 1: Toxicity to the kidney.

1-Methyl-cyclopropene	Fenpropidin	Pethoxamid
2,4-D	Fenpyroximate	Phenmedipham
2,4-DB	Fipronil	Picloram
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Flazasulfuron	Pirimiphos-methyl
Acetamiprid	Florasulam	Prohexadione-calcium
Aclonifen	Fludioxonil	Propamocarb
Azimsulfuron	Flufenacet (formerly fluthiamide)	Propaquizafop
Beflubutamid	Flumioxazin	Propyzamide
Benfluralin	Fluopicolide	Prosulfocarb
Bentazone	Fluoxastrobin	Prothioconazole

Benthiavalicarb	Fluroxypyr	Pyraclostrobin
Benzoic acid	Folpet	Pyraflufen-ethyl
Beta-Cyfluthrin	Forchlorfenuron	Pyrimethanil
Bifenox	Fosetyl	Pyriproxyfen
Carbendazim	Gibberellin	Quinoclamine
Chloridazon (aka pyrazone)	Glufosinate	Quizalofop-P-tefuryl
Chlorothalonil	Glyphosate	Rimsulfuron (aka renriduron)
Chlorotoluron	Ioxynil	S-Metolachlor
Chlorpropham	Iprodione	Sodium 5-nitroguaiacolate
Cinidon ethyl	Isoxaflutole	Sodium o-nitrophenolate
Clodinafop	Linuron	Sodium p-nitrophenolate
Clopyralid	Lufenuron	Spinosad
Clothianidin	MCPA	Spiroxamine
Copper compounds	Mecoprop	Sulcotrione
Cyazofamid	Mecoprop-P	Sulfosulfuron
Cyflufenamid	Mepanipyrim	Tebufenpyrad
Cyhalofop-butyl	Mesotrione	Tepraloxydim
Cymoxanil	Metamitron	Tetraconazole
Cyprodinil	Metazachlor	Thiabendazole
Cyromazine	Metconazole	Thiamethoxam
Desmedipham	Methomyl	Thiophanate-methyl
Dichlorprop-P	Methoxyfenozide	Tolylfluanid
Dimethachlor	Metrafenone	Tri-allate
Diquat (dibromide)	Metribuzin	Triasulfuron
Diuron	Milbemectin	Tribenuron methyl
Esfenvalerate	Molinate	Triclopyr
Ethephon	Oxadiazon	Trifloxystrobin
Etofenprox	Oxamyl	Trinexapac (aka cimetacarb ethyl)
Fenhexamid	Oxasulfuron	Tritosulfuron
Fenoxaprop-P	Penconazole	

16.2.2. CAG level 2: Phenomenological / specific effects on the kidney

Various types of effects on the kidney were identified as a basis for establishing CAGs at level 2. Based on these effects, thirteen distinct CAGs at level 2 are proposed: Tubular hypertrophy / hyperplasia, tubular fatty changes, tubular degeneration / death, tubular neoplasms, tubular hyaline droplets, chronic progressive nephropathy (CPN), alpha2u-globulin nephropathy, glomerular degeneration / death, glomerular inflammation, inflammation, papillary degeneration / death, papillary hypertrophy / hyperplasia, and pelvic hyperplasia. More information is given in Appendix U.

16.2.2.1. CAG level 2a: Tubular cell degeneration / cell death

This CAG level covers effects described as tubular nephropathy, tubular degeneration, tubular death, and tubular atrophy. Tubular nephropathy is unspecified damage to the tubular cells. Degeneration of cells reflects cytoplasmic alterations at the borderline between adaptation with resolution back to normal structure and function, and inability to adapt leading to cell death. It may be difficult to distinguish between different forms of degeneration, and early

necrosis. Cell death is the ultimate result of irreversible cellular injury. Tubular atrophy represents collapse of tubular structures, ultimately with disappearance and reduction of individual tubules (Hard et al 1999).

Effects described by a term such as chronic nephropathy is included in this CAG at least if the effect occurred in other species than rats or in subchronic studies. For long-term rat studies the matter is more complicated as the DARs by the term chronic nephropathy may refer to CPN (see CAG level 2f). Chronic nephropathy in principle also may include damage to the glomerulus or other parts of the kidney. However, if the lesions in the chronic nephropathy have not been specified by the DARs they have just been included in this CAG.

See Annex U for a list of all terms in the DARs interpreted to represent tubular cell degeneration / cell death. CAGs have not been created for damage to specific sites within the tubules as this information rarely has been noted in the DARs.

The active substances identified as inducing tubular cell degeneration / cell death are allocated to CAG level 2a, termed ‘CAG level 2a: Tubular cell degeneration / cell death’ and are listed in Table 16.2.

Table 16.2. CAG level 2a: Tubular cell degeneration / cell death

2,4-D	Fludioxonil	Pyraclostrobin
2-Phenylphenol	Flufenacet	Pyrimethanil
Aclonifen	Fluoxastrobin	Pyriproxyfen
Carbendazim	Fluroxypyr	Quinoclamine
Chloridazon (aka pyrazone)	Forchlorfenuron	Sodium 5-nitroguaiacolate
Chlorothalonil	MCPA	Sodium o-nitrophenolate
Cinidon ethyl	Mecoprop-P	Sodium p-nitrophenolate
Copper compounds	Mepanipyrim	Spinosad
Cyhalofop-butyl	Metazachlor	Sulfosulfuron
Cyromazine	Metrafenone	Tetraconazole
Dichlorprop-P	Oxasulfuron	Thiabendazole
Dimethachlor	Pethoxamid	Thiamethoxam
Etofenprox	Pirimiphos-methyl	Thiophanate-methyl
Fenhexamid	Propaquizafop	Triasulfuron
Fenoxaprop-P	Propyzamide	Triclopyr
Flazasulfuron	Prosulfocarb	Tritosulfuron
Florasulam	Prothioconazole	

16.2.2.2. CAG level 2b: Tubular fatty changes

This CAG level covers effects described as fatty changes and/or vacuolation. Tubular vacuolation is recognised as clear, round spaces of variable size within the cytoplasm. Vacuolation may be a transient physiological response or it may represent the initial stage preceding cell degeneration. The vacuoles may or may not contain accumulated fat. (Hard et al. 1999).

Most of the DARs do not detail the content of the vacuoles. Consequently it is probably not all the active substances included in this CAG that actually contain fat in the vacuoles.

See Annex U for a list of all terms in the DARs interpreted to represent tubular fatty change.

The active substances identified as inducing fatty change are allocated to CAG level 2b and are listed in Table 16.3.

Table 16.3. CAG level 2b: Tubular fatty changes

2,4-D	Metazachlor	Prosulfocarb
Chloridazon (aka pyrazone)	Metconazole	Spinosad
Cyflufenamid	Oxadiazon	Thiabendazole
Dimethachlor	Oxamyl	Triclopyr
Fenoxaprop-P	Pethoxamid	Tritosulfuron
Flufenacet	Prohexadione-calcium	

For twelve out of the seventeen active substances, which induce tubular fatty changes, tubular cell degeneration / cell death has also been noted.

16.2.2.3. CAG level 2c: Tubular hypertrophy / hyperplasia

Tubular hypertrophy / hyperplasia covers tubular hypertrophy, tubular regeneration, and tubular hyperplasia. Tubular hypertrophy is an increase in the size of the epithelial cells lining the renal tubules. It may develop after tubule cell necrosis as a compensatory response to transient loss of tubule function. Regenerating tubular epithelium is characterised by basophilia and a higher than normal proliferative rate. Tubule regeneration is the epithelial response to tubular necrosis. Tubular hyperplasia is an increase in the number of epithelial cells lining the renal tubules. It may develop following longstanding persistent tubule regeneration. (Hard et al. 1999).

Tubules may be basophilic unrelated to regeneration. It is likely that chemically induced cytoplasmic basophilia in tubules is a reflection of an increase in ribosomal RNA. Basophilic tubules also characterise the early stages of CPN (see CAG level 2f). (Hard et al. 1999). Often it was not clear in the DARs whether the basophilic tubules were considered to be regenerating tubules, tubules in the early stage of CPN, or tubules unrelated to regeneration or CPN. In those cases the basophilic tubules have been included in this CAG for hypertrophy / hyperplasia.

See Annex U for a list of all terms in the DARs interpreted to represent tubular hypertrophy / hyperplasia.

The active substances identified as inducing tubular hypertrophy / hyperplasia are allocated to CAG level 2c and are listed in Table 16.4.

Table 16.4. CAG level 2c: Tubular hypertrophy / hyperplasia

2,4-D	Fenhexamid	Prothioconazole
2-Phenylphenol	Florasulam	Quinoclamine
Aclonifen	Fluroxypyr	Sodium 5-nitroguaiacolate
Chlorothalonil	Forchlorfenuron	Sodium o-nitrophenolate
Cinidon ethyl	MCPA	Sodium p-nitrophenolate
Copper compounds	Metconazole	Sulcotrione
Cyazofamid	Oxadiazon	Tetraconazole
Cyromazine	Oxamyl	Thiabendazole
Desmedipham	Pethoxamid	Tolylfluanid
Dichlorprop-P	Prohexadione-calcium	Triasulfuron
Etofenprox	Propaquizafop	Triclopyr

For 25 out of the 33 active substances, which induce tubular hypertrophy / hyperplasia, tubular cell degeneration / cell death has also been noted. For 3 out of the 8 active substances that did not induce tubular cell degeneration / cell death, CPN was noted.

16.2.2.4. CAG level 2d: Tubular neoplasms

For the purpose of the CAG project, histopathological findings in the form of tubular adenoma and tubular carcinoma are interpreted, qualitatively but not quantitatively, as representing the same type of effect in the kidney and therefore, are allocated to a single CAG level 2, termed ‘CAG level 2d: Tubular neoplasms’. See Annex U for a list of all terms in the DARs interpreted to represent tubular neoplasms.

The active substances identified as inducing tubular neoplasms are allocated to CAG level 2d and are listed in Table 16.5.

Table 16.5. CAG level 2d: Tubular neoplasms

Benfluralin	Chlorotoluron	Molinate
Chlorothalonil	Forchlorfenuron	

16.2.2.5. CAG level 2e: Tubular hyaline droplets

Hyaline droplets are lysosomes containing protein (Hard et al. 1999). Hyaline droplets solely in male rats may be part of alpha2u-globulin nephropathy (see CAG level 2g) and is not included in CAG level 2e. See Annex U for a list of all terms in the DARs interpreted to represent tubular hyaline droplets.

The active substances identified as inducing tubular hyaline droplets in both sexes of rats or in other species are allocated to CAG level 2e and are listed in Table 16.6.

Table 16.6. CAG level 2e: Tubular hyaline droplets

Azimsulfuron	Benthiavalicarb	Mesotrione
Benfluralin	Copper compounds	

16.2.2.6. CAG level 2f: Chronic progressive nephropathy (CPN)

CPN is a common spontaneous disease of aging rats of unknown etiology. It occurs in both sexes but is more prevalent and more severe in male rats. Several physiological factors influence its incidence and severity, most prominently protein and caloric intake. Chemicals may also exacerbate CPN. In its early stages it has histological similarity to tubular degeneration from other causes. In its advanced stages it is associated with marginally increased renal tubule tumour incidences. The earliest change is foci of basophilic tubules with thickened basement membrane and hyaline casts. The first lesions may develop as early as 2-3 months in conventional strains of laboratory rats. With progression affected tubules display both degenerative and regenerative changes including hyperplasia. Dilated tubules are filled with hyaline casts. With increasing severity tubular atrophy and glomerular changes become evident. The glomerular changes include collapse of capillary tufts, adhesions with Bowman´s capsule, glomerular atrophy and sclerosis. Interstitial changes include inflammation and fibrosis. In very advanced stages, little normal parenchyma remains in the cortex, the tubules are cystic and there may be mineralisation of the basement membranes (because of compensatory hyperplasia of the parathyroid gland with general mineralisation). Chemically induced exacerbation of CPN and increases in the incidence of CPN-related renal tumours is not considered relevant to humans. (Hard et al. 2009, Hard and Khan 2004, Hard et al. 1999). Most chemicals that induce alpha2u-globulin nephropathy also exacerbate CPN in male rats but no evidence suggests that the high level of alpha2u-globulin in male rats has any influence on the expression of CPN. It is however possible that the two modes of action, alpha2u-globulin and CPN, may work in concert to enhance the formation of renal tubule tumours. (Hard et al. 2009).

Recently Hard and Seely (2005) have recommended criteria to assist pathologists in the discrimination between proliferative lesions found in advanced CPN and preneoplastic lesions. According to Hard et al. (1999), CPN should be identified by this term in safety evaluation studies and the lesion components of CPN should not be individually diagnosed. So it should be easy to go through the DARs and find the active substances that may exacerbate CPN. However, in practice the terms chronic progressive nephropathy or CPN have rarely been used in the DARs. Over the years CPN has been called several names e.g. glomeruloschlerosis, glomerulonephritis and chronic nephrosis (Hard et al. 2009). Sometimes the DARs specify the lesions covered e.g. by the term chronic nephropathy but just as often the terms are not specified. Consequently, it is not always clear whether the DARs mean CPN. See Annex U for a list of all terms in the DARs interpreted to represent CPN. In general, active substances have only been included in this CAG if CPN was noted in long time studies with rats. Some of the effects seen in the 90-day studies may be early indications of CPN but it is not clear from the DARs whether that is the case. For at least one active substance, dimethachlor, the DAR has used the term chronic progressive nephropathy for effects seen in

mouse studies. Hard et al. (2009) shortly mention that mice can develop a form of CPN that bears some resemblance to rat CPN. However, dimethachlor is not included in this CAG as it is not clear from the articles whether CPN in mice is without relevance for humans.

The active substances identified as inducing CPN are allocated to CAG level 2f and are listed in Table 16.7.

Table 16.7. CAG level 2f: Chronic progressive nephropathy (CPN)

Beflubutamid	Flumioxazin	Picloram
Benfluralin	MCPA	Pirimiphos-methyl
Benthiavalicarb	Mesotrione	Prothioconazole
Chlorothalonil	Metazachlor	Sulcotrione
Cinidon ethyl	Metconazole	Thiamethoxam
Clodinafop	Methoxyfenozide	Thiophanate-methyl
Fipronil	Metrafenone	Tri-allate
Flazasulfuron	Milbemectin	
Fludioxonil	Oxadiazon	

16.2.2.7. CAG level 2g: Alpha2u-globulin nephropathy

Alpha2u-globulin nephropathy (where u is short for urinary) is a renal syndrome that occurs exclusively in male rats. The toxicity appears to be caused by the accumulation of the protein, alpha2U-globulin. The protein overload causes renal cell injury, compensatory cell proliferation and ultimately a low incidence of renal tubule tumours. Alpha2u-globulin is a protein that is only synthesised by adult male rats. Chemicals that cause alpha2u-globulin nephropathy (or their metabolites) bind to alpha2u-globulin. Because of this binding the rate of degradation of alpha2u-globulin is reduced and the protein begins to accumulate in the phagolysosomes. This accumulation of protein is histopathologically seen as hyaline droplets in the proximal tubular cells. Continued exposure to the chemical leads to single cell degeneration and necrosis in the tubular cells. Dead cells are sloughed into the tubular lumen and contribute to the development of granular casts. After several months of exposure, mineralisation occurs in the lumen of tubules in the renal papilla often accompanied by papillary hyperplasia. The mineralisation represents residues of the granular casts formed in more active stages of injury. As a result of the renal cell death and degeneration, tubular hyperplasia and ultimately tubular neoplasms may develop. There is no protein in humans that can contribute to a renal syndrome like alpha2u-globulin nephropathy. Thus alpha2u-globulin nephropathy and increases in the incidence of renal tumours related to alpha2u-globulin nephropathy is not considered relevant to humans. (Swenberg and Lehman-McKeeman 1999, Hard et al. 1999).

IARC 1999 has developed criteria that need to be met to conclude that a chemical causes kidney tumours through an alpha2u-globulin mode of action.

In this CAG for alpha2u-globulin nephropathy active substances, which induce hyaline droplets and sometimes one or more of the other characteristic lesions (single cell

degeneration and necrosis, granular casts, papillary mineralisation, tubular hyperplasia, tubular neoplasms) for alpha2u-globulin nephropathy only in male rats have been included. Thus the active substances in this CAG do not necessarily meet the IARC 1999 criteria (actually none of them do).

The active substances identified as inducing alpha2u-globulin nephropathy are allocated to CAG level 2g and are listed in Table 16.8.

Table 16.8. CAG level 2g: Alpha2u-globulin nephropathy

1-Methyl-cyclopropene	Fluopicolide	Thiamethoxam
Benfluralin	Prosulfocarb	Tri-allate
Cyflufenamid	Quinoclamine	Trinexapac
Flazasulfuron	Tepraloxydim	

In the remarks column in the database it is noted which characteristic lesions the 11 active substances induce. Shortly 5 of the active substances (1-methyl-cyclopropene, benfluralin, cyflufenamid, flazasulfuron, and quinoclamine) only induce hyaline droplets. Thiamethoxam, tri-allate, and trinexapac induce hyaline droplets, granular casts, and tubular hyperplasia. Tepraloxydim induces hyaline droplets and single cell degeneration and necrosis. Prosulfocarb induces hyaline droplets, single cell degeneration and necrosis, and tubular hyperplasia. Fluopicolide as the only active substance induces almost the whole sequence of lesions (hyaline droplets, single cell degeneration and necrosis, granular casts, papillary mineralisation, and tubular hyperplasia). Only benfluralin induces tubular neoplasms but clearly not through an alpha2u-globulin mode of action (see the CAG for tubular neoplasms for further details).

16.2.2.8. CAG level 2h: Glomerular cell degeneration / cell death

The CAG level for glomerular degeneration / death mainly covers glomeruloschlerosis. Glomerulosclerosis represent progressive replacement of glomerular matrix with amorphous hyaline material. Early changes include a thickened basement membrane. Glomeruloschlerosis in rats is a component of advanced CPN but can also be induced experimentally by the administration of chemicals. (Hard et al. 1999). To complicate matter further, CPN has over the years been called several names of which glomeruloschlerosis is one (Hard et al. 2009). See Annex U for a list of all terms in the DARs interpreted to represent glomerular degeneration / death.

The active substances identified as inducing glomerular degeneration / death are allocated to CAG level 2h and are listed in Table 16.9.

Table 16.9. CAG level 2h: Glomerular cell degeneration / cell death

Benthiavalicarb	Ethephon	Molinate
Carbendazim	Fludioxonil	Propamocarb
Chlorothalonil	Fluroxypyr	Propaquizafop

For two of the substances, benthiavalicarb and ethephon, the effect was noted in 2-year rat studies, and thus may represent CPN.

16.2.2.9. CAG level 2i: Glomerular inflammation

The CAG level for glomerular inflammation covers 2 active substances, which induce the effects glomerulonephritis or membranous glomerulonephritis in non-rat studies. In membranous glomerulonephritis, the glomerular basement membranes are thickened due to deposition of aggregates of immune complexes (Hard et al. 1999). To complicate matter further, CPN has over the years been called several names of which glomerulonephritis is one (Hard et al. 2009). Thus a few active substances where the terms glomerulonephritis or progressive glomerulonephritis have been used in the DARs for effects in long term rat studies have been allocated to the CAG for CPN.

The active substances identified as inducing glomerular inflammation are allocated to CAG level 2i and are listed in Table 16.10.

Table 16.10. CAG level 2i: Glomerular inflammation

Chlorothalonil	Sulfosulfuron	

16.2.2.10. CAG level 2j: Inflammation

Infiltration of different inflammatory cells is typically a response to death of cells. Thus tubular or papillary necrosis may cause inflammation. Calculi in the pelvis may also be the cause of an inflammation in the kidney. In rats an infection with bacteria may easily ascend from the bladder to the kidney and cause inflammation. Inflammation between the tubules is common in advanced stages of CPN. (Hard et al. 1999). See Annex U for a list of all terms in the DARs interpreted to represent inflammation.

The active substances identified as inducing inflammation are allocated to CAG level 2j and are listed in Table 16.11.

Table 16.11. CAG level 2j: Inflammation

2-Phenylphenol	Fludioxonil	Pyrimethanil
Aclonifen	Fluroxypyr	Quinoclamine
Azimsulfuron	Forchlorfenuron	Spinosad
Benfluralin	Fosetyl	Sulfosulfuron
Benthiavalicarb	Iprodione	Thiabendazole
Bifenox	Lufenuron	Thiamethoxam
Carbendazim	Oxamyl	Triasulfuron
Chloridazon (metabolite)	Oxasulfuron	Tritosulfuron

Chlorothalonil	Prothioconazole	
Desmedipham	Pyraflufen-ethyl	

Calculi, tubular necrosis or papillary necrosis have been noted for almost all of the active substances, which induce inflammation. Thus for most of the active substances the inflammation is probably secondary to effects already covered in other CAGs.

16.2.2.11. CAG level 2k: Papillary cell degeneration / cell death

Papillary necrosis starts at the tip of the papilla and may progress to involve the full depth of the papilla. At first the degenerative change involves the interstitial cells and blood vessels. Later the degenerative change involves the tubules. The demarcation between necrotic papilla and the surviving medullary tissue may show infiltration of inflammatory cells and mineralisation. The necrotic papilla may undergo regeneration, which may result in papillary hyperplasia. Secondary changes of tubular dilation and inflammation may occur in the cortex. (Hard et al. 1999). See Annex U for a list of all terms in the DARs interpreted to represent papillary cell degeneration / cell death.

The active substances identified as inducing papillary cell degeneration / cell death are allocated to CAG level 2k and are listed in Table 16.12.

Table 16.12. CAG level 2k: Papillary cell degeneration / cell death

2-Phenylphenol	Fosetyl	Quinoclamine
Aclonifen	Metamitron	Sulcotrione
Benfluralin	Picloram	Tetraconazole
Florasulam	Propaquizafop	Tritosulfuron
Fluroxypyr	Pyraflufen-ethyl	

16.2.2.12. CAG level 2l: Papillary hypertrophy / hyperplasia

As described above papillary hyperplasia may be a regenerative response to papillary necrosis (Hard et al. 1999). See Annex U for a list of all terms in the DARs interpreted to represent papillary hypertrophy / hyperplasia.

The active substances identified as inducing papillary hypertrophy / hyperplasia are allocated to CAG level 2l and are listed in Table 16.13.

Table 16.13. CAG level 2l: Papillary hypertrophy / hyperplasia

2-Phenylphenol	Fluopicolide	Pyraflufen-ethyl
Chlorothalonil	Fluroxypyr	Quinoclamine
Florasulam	Phenmedipham	Thiabendazole

Five of the active substances (2-phenylphenol, florasulam, fluroxypyr, pyraflufen-ethyl, and quinoclamine) that induce papillary hypertrophy / hyperplasia also induce papillary degeneration / death. Thus for these five substances the hyperplasia is probably secondary to the papillary necrosis.

Six of the active substances (2-phenylphenol, chlorothalonil, fluroxypyr, phenmedipham, pyraflufen-ethyl, quinoclamine and thiabendazole) that induce papillary hypertrophy / hyperplasia also induce pelvic hyperplasia. It makes one speculate whether the cause of the papillary hyperplasia at times may be related to the cause of the pelvic hyperplasia.

16.2.2.13. CAG level 2m: Pelvic hyperplasia

Hyperplasia is an increased number of cells. Hyperplasia of the pelvis may be a response to epithelial irritation, which in turn can be induced by urinary crystals, calculi or toxic chemicals (Cohen 1998, Hard et al. 1999). See Annex U for a list of all terms in the DARs interpreted to represent pelvic hyperplasia.

The active substances identified as inducing pelvic hyperplasia are allocated to CAG level 2m and are listed in Table 16.14.

Table 16.13. CAG level 2m: Pelvis hyperplasia

2,4-D	Desmedipham	Methoxyfenozide
2-Phenylphenol	Diuron	Oxasulfuron
Aclonifen	Flazasulfuron	Phenmedipham
Azimsulfuron	Flufenacet	Pyraflufen-ethyl
Benfluralin	Fluoxastrobin	Quinoclamine
Benthiavalicarb	Fluroxypyr	Spiroxamine
Chloridazon (metabolite)	Fosetyl	Sulfosulfuron
Chlorothalonil	Glyphosate (metabolite)	Thiabendazole
Clothianidin	Linuron	Thiamethoxam
Cyromazine	Mepanipyrim	Triasulfuron

16.2.2.14. Effects not considered relevant for CAGs at level 2

Changes in blood and urine parameters indicative of kidney damage:

Initially, nephrotoxicity can be assessed by evaluating serum chemistry and urinalyses following treatment with the toxicant. The standard battery of tests includes measurement of urine volume and osmolality, pH, and urinary composition (e.g. electrolytes, glucose, protein). The specificity of the tests often is poor. As examples, glucosuria may reflect chemically induced defects in the proximal tubules but may also be secondary to hyperglycemia. Urinary excretion of high molecular weight proteins such as albumin is suggestive of glomerular injury, whereas excretion of low molecular weight proteins suggests proximal tubular damage. Enzymes in the urine may reflect damage to more or less specific sites in the kidney (or in other organs). The absence of enzymuria does not necessarily reflect an absence of damage, as

it is often a transient phenomenon. Blood urea nitrogen (BUN) and/or creatinine are often measured in blood. These two substances are waste products that are normally excreted by the kidneys. Increases in the blood of BUN or creatinine suggest decreases in the glomerular filtration rate (GFR). However, both substances are rather insensitive indices of the GFR as a 50 to 70 percent decrease in GFR must occur before increases in blood BUN and/or creatinine develop. Increases in BUN and /or creatinine may not necessarily reflect renal damage but rather may be secondary to dehydration, hypovolemia, and/or protein catabolism. (Klaassen 1996).

Changes in all of the above parameters thus are more or less specific indicators for damage to the kidney. However, the CAGs for different histopathological effects will cover the kidney injury indicated by these blood and urine parameters.

Hypertrophy (collecting ducts):

Florasulam induces various effects in the kidneys including tubular and papillary hypertrophy / hyperplasia. In addition in most short and long term studies hypertrophy of the collecting ducts have been reported. The DAR speculates on possible mode of actions for the hypertrophy of the collecting ducts. Anyhow as florasulam is the only substance where hypertrophy of the collecting ducts have been noted and the possible mode of actions are speculations, a CAG has not been created for the effect, which is considered covered by either tubular or papillary hypertrophy / hyperplasia.

Tubular karyomegaly:

Tubular karyomegaly is an increase in the nuclear size of the tubular cells. The pathogenesis is not known but the presence generally implies a response to a variety of toxic agents. (Hard et al. 1999). Chlorothalonil is the only active substance where tubular karyomegaly have been noted in several studies. Chlorothalonil induces various other effects in the tubular cells (degeneration / death, hypertrophy / hyperplasia, and neoplasms). Thus the tubular karyomegaly is considered covered by other CAGs for tubular effects.

Tubular dilation:

Tubular dilation covers tubular dilation and tubular cysts. Tubular dilation is an increase in lumen diameter of the tubules. It may be induced e.g. by luminal obstruction by crystalline deposits, tubular hyperplasia, inflammation, or tubule cell degeneration/regeneration. Tubular cysts are marked focal distension of tubules. Causative factors are poorly understood, but tubular cysts may develop secondary to tubule obstruction by casts or interstitial fibrosis. (Hard et al. 1999). Thus tubular dilation is considered secondary to the effect already covered by other CAGs especially the CAGs for tubular degeneration / death, tubular hypertrophy / hyperplasia, and inflammation.

Tubular casts:

Tubular casts cover hyaline casts and granular casts. Hyaline casts are homogeneous matter in the tubule lumen typically composed of protein. They are associated with increased protein in the glomerular filtrate, such as albumin, or with secretion into the lumen from tubule cells. Hyaline casts are a characteristic of CPN (see CAG level 2f). Granular casts are non-homogenous particulate matter in the tubule lumen presenting cell breakdown products and debris. Typically they occur secondary to tubular necrosis or apoptosis, as in alpha2u-globulin nephropathy (see CAG level 2g). (Hard et al. 1999). Thus tubular casts are considered secondary to the effect already covered by other CAGs especially the CAGs for tubular degeneration / death, CPN and alpha2u-globulin nephropathy.

Pelvic metaplasia:

The epithelial cells are transformed into squamous epithelial cells typically as a response to irritation. Pelvic metaplasia has only been noted for 2 active substances (fluroxypyr and sulfosulfuron) where pelvic hyperplasia also was noted. Although pelvic metaplasia is not the same as pelvic hyperplasia, the pelvic metaplasia is considered covered by the CAG level 2m for pelvic hyperplasia.

Pelvic cell degeneration / cell death:

As the only active substance fluroxypyr induce pelvic degeneration / death. Fluroxopyr also induce glomerular, tubular and papillary degeneration / death, and in the pelvis hyperplasia and metaplasia have been noted. Thus the pelvic degeneration / death is considered covered by some of the other CAGs.

Pelvic dilation:

Pelvic dilation is dilation of the renal pelvis and calyces. Pelvic dilation may be secondary to urinary obstruction e.g. because of calculi. (Hard et al. 1999). Thus pelvic dilation is considered secondary to the effects already covered by other CAGs especially the CAG level 3m1 for calculi.

Ureter effects:

Ureter effects cover epithelial cell death, hyperplasia, metaplasia and dilation. Only 4 active substances (benfluralin, chloridazon, fosetyl, and sulfosulfuron) induce effects on the ureters. Calculi have been found and pelvic hyperplasia noted for all 4 active substances. In addition 3 of the substances also induce hyperplasia in the bladder and two of them neoplasms in the bladder. Thus the effects on the ureters are considered covered by other CAGs especially by the CAG level 2m: Pelvic hyperplasia, but also by CAGs for effects on the urinary bladder.

Mineralisation:

Mineralisation in the rat kidney mainly represents calcium salt deposition. The spontaneous occurrence often depends on the calcium-phosphorous ratio in the diet. Mineralisation may

occur in different locations in the kidney. Tubular mineralisation is usually a consequence of tubular degeneration. Papillary mineralisation may occur as part of the alpha2u-globulin nephropathy or may be secondary to papillary necrosis. Mineralisation may also occur in the pelvis. In renal failure e.g. in end-stage CPN, generalised mineralisation may occur because of compensatory hyperplasia of the parathyroid glands and release of calcium from the bones. (Hard et al. 1999). Thus mineralisation often seems to be secondary to effects already covered in other CAGs.

Fibrosis:

Fibrosis is the formation of excess connective tissue in a reaction to toxicity of the kidney. Fibrosis in the kidney has been found for about 15 substances. Fibrosis in the kidney is considered secondary to the effects already covered by other CAG´s especially the CAG level 2a: Tubular degeneration / death.

Pigmentation:

The most commonly encountered pigments are hemosiderin, bilirubin or lipofuscin (Hard et al. 1999). Hemosiderin is precipitated iron following for instance hemolytic anemia. Bilirubin is a byproduct of destruction of (aged) red blood cells. An increased amount of bilirubin may appear in the kidneys following hepatic dysfunction. Lipofuscin represent a breakdown of cell membranes, and is often observed in aging rats. Thus pigmentation is either an indirect effect (hemosiderin, bilirubin) or should be covered by the CAG for tubular degeneration / death (lipofuscin).

16.2.3. CAG level 3: Mode of action

For some of the phenomenological / specific effects on the kidney described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

16.2.3.1. CAG level 3g1: Alpha2u-globulin

One of the criteria that need to be met to conclude that a chemical causes kidney tumours through an alpha2u-globulin mode of action is that alpha2u-globulin is identified as the accumulating protein in tubule cells (IARC 1999).

The active substances identified as increasing alpha2u-globulin in male rats are allocated to CAG level 3g1 and are listed in Table 16.16.

Table 16.16. CAG level 3g1: Alpha2u-globulin

Benfluralin	Flazasulfuron	Tri-allate
Cyflufenamid	Thiamethoxam	

The standard toxicological guidelines do not include as a mandatory requirement analysis of alpha2u-globulin in tubule cells. Therefore, more active substances than those listed may induce accumulation of alpha2u-globulin.

16.2.3.2. CAG level 3m1: Calculi

Calculi are stones. Calculi in the kidney usually occur in the renal pelvis either attached to the epithelium or lying free in the pelvis (Hard et al 1999). In the bladder calculi may cause erosion and ulceration of the epithelium with hemorrhage, inflammation and regeneration. Severe damage to the epithelium may lead to hyperplasia and ultimately neoplasms. The pathogenesis appears to be similar for the renal pelvis (and ureter). Formation of calculi requires the concentration of the critical substances (e.g. active substance, metabolite, calcium) in the urine to be sufficiently high to lead to precipitate formation and ultimately to calculi. This can be influenced by specific chemical and physical condition of the urine (e.g. pH, volume). (Cohen 1998). Calculi in the pelvis, ureters or bladder may cause obstruction of the renal flow, which may lead to pelvic (and tubular dilation) and ultimately to damage to the cells in the kidney. See Annex U for a list of all terms in the DARs interpreted to represent calculi.

The active substances identified as inducing calculi are allocated to CAG level 3m1 and are listed in Table 16.17.

Table 16.17. CAG level 3m1: Calculi

2,4-D	Chloridazon (metabolite)	Linuron
2-Phenylphenol	Clothianidin	Oxasulfuron
Benfluralin	Desmedipham	Sulfosulfuron
Benthiavalicarb	Fluoxastrobin	Thiabendazole
Bifenox	Fosetyl	Triasulfuron

Calculi frequently are passed during the course of an experiment by the animals without being detected by the investigators because they are too small for gross visual detection or they have dissolved in the urine (Cohen 1998). Therefore, more active substances than those listed may cause pelvic hyperplasia by a calculi mode of action.

All of the 15 active substances, which induce calculi, also induce pelvic hyperplasia - except one (bifenox). Most of these active substances also induce inflammation of the kidney (2-phenylphenol, benfluralin, bifenox, chloridazon (metabolite), desmedipham, fosetyl, oxasulfuron, sulfosulfuron, thiabendazole, and triasulfuron).

Even though studies with 2-phenylphenol have shown calculi in the bladder and in the kidney, calculi does not seem to be considered part of the mode of action for 2-phenylphenol by the DAR. For fluoxastrobin and fosetyl, calculi have been discussed as part of the mode/mechanism of action for these substances (see CAG level 4m). For benfluralin the DAR

suggests that calculi is the cause of the pelvic hyperplasia. For the rest of the active substances, which induce calculi, no mode/mechanism of action for the pelvic hyperplasia was discussed in the DARs.

Bifenox do not induce pelvic hyperplasia but inflammation. The DAR suggests that the calculi may have caused obstruction to renal flow, which subsequently led to infection.

16.2.3.3. CAG level 3m2: Crystals

Like calculi, crystals in the urine may lead to erosion of the epithelium in the pelvis, ureters and bladder that may result in regenerative hyperplasia and ultimately neoplasms usually without inflammation. (Cohen 1998). See Annex U for a list of all terms in the DARs interpreted to represent crystals.

The active substances identified as inducing crystals are allocated to CAG level 3m2 and are listed in Table 16.18.

Table 16.18. CAG level 3m2: Crystals

Aclonifen	Flazasulfuron	Prothioconazole
Azimsulfuron – in urinary bladder	Fluoxastrobin – in urinary bladder	Sulfosulfuron - in urinary bladder
Chloridazon (metabolite) – in urinary bladder	Iprodione	

Azimsulfuron, chloridazon (metabolite), fluoxastrobin, and sulfosulfuron have been included in CAG level 3m2 even though crystals have not been detected in the kidney but only in the bladder as the substances clearly have the potential to produce crystals.

Six of the 8 active substances, which induce crystals, induce hyperplasia of the pelvis (aclonifen, azimsulfuron, chloridazon (metabolite), flazasulfuron, fluoxastrobin, and sulfosulfuron). Three of the substances also induce calculi (chloridazon (metabolite), fluoxastrobin, and sulfosulfuron).

16.2.3.4. CAG level 3n1: Oxidative stress

Oxidative stress occurs in cells when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability. ROS can be produced in normal cellular metabolism or by inflammatory cells. Reactive intermediate metabolites produced in the metabolism of xenobiotics may enhance the formation of ROS. Antioxidants such as vitamin C, vitamin E, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase normally inactivate ROS. With excessive formation of reactive intermediate metabolites and ROS the antioxidant capacity may be overloaded. The result is oxidative stress, which may result in damage to DNA, lipids, and proteins in the cell. Unrepaired DNA damage may lead to new mutations and potentially tumours. Oxidative injury may also produce cell death, which may lead to regenerative hyperplasia and ultimately tumours. (Klaunig et al. 1998).

Active substances, which induce oxidative stress measured as a decreased level of the antioxidant GSH are included in this CAG level.

The active substances identified as inducing oxidative stress are allocated to CAG level 3n1 and are listed in Table 16.15.

Table 16.15. CAG level 3n1: Oxidative stress

2-Phenylphenol		

The standard toxicological guidelines do not include as a mandatory requirement studies on oxidative stress. Therefore, more active substances than those listed may cause kidney toxicity by an oxidative stress mode of action.

16.2.3.5. Other information reported

Mode of action for kidney tumours:

Lock and Hard 2004 have categorized 69 renal carcinogens based on mechanistic information. They have created the following categories that were based on the categories identified in IARC 1999:

- Direct interaction of the chemical or metabolite with renal DNA
- Indirect interaction with DNA mediated by oxidative stress leading to increased production of reactive oxygen species
- Conjugation with gluthathion (GSH) and subsequent enzymatic activation to a reactive species
- Sustained stimulation of tubule cell proliferation in response to cytotoxicity caused directly by the chemical
- Sustained stimulation of tubule cell proliferation by indirect cytotoxicity involving hyaline droplets and alpha2u-globulin accumulation
- Chemically induced exacerbation of chronic progressive nephropathy (CPN).

As the DARs consider all the active substances non-genotoxic, none of the 5 active substances, which induce tumours in the kidney, act through the first mode of action. No mechanistic studies have been located on the second mode of action.

As increases in the incidence of renal tumours related to alpha2u-globulin nephropathy are not considered relevant to humans, IARC 1999 has developed criteria that need to be met to conclude that a chemical causes kidney tumours through an alpha2u-globulin mode of action. Some of the criteria that need to be met are that the nephropathy and tumours are specific to the male rat, that the characteristic sequence of histopathological changes are induced, and that alpha2u-globulin is identified as the accumulating protein in tubule cells.

As increases in the incidence of CPN-related renal tumours are not considered relevant to humans, criteria have also been developed for CPN as the underlying cause of tubular

tumours. First and foremost the chemical must have been shown to exacerbate CPN to very advanced stages of severity. Secondly the tumours must be of low incidence and predominantly adenomas of small size. The tumours must be restricted to the CPN-affected tissue. Finally the parts of the kidney not affected by CPN should reveal no evidence of compound-induced cellular injury that would suggest an alternative mode of action. (Lock and Hard 2004, Hard and Khan 2004).

For two of the active substances (chlorotoluron and molinate) tumours are the only effect in the kidney in the long term studies. For chlorotoluron one of the DAR addenda speculates that kidney tumours may be related to excessive proliferation secondary to cytotoxicity resulting from the excretion of toxic glutathione related conjugates. However, neither tubular cytotoxicity nor tubular regeneration is described to occur in the study where tumours were noted, and no mechanistic studies seem to have been performed. For molinate the DAR speculates that the tumours may be caused by an alpha2u-globulin nephropathy as the tumours are only noted in male rats. However, none of the characteristic lesions of alpha2u-globulin nephropathy are described to occur in the study.

For chlorothalonil and forchlorfenuron the most likely mode of action is cytotoxicity as tubular degeneration / death and tubular hypertrophy / hyperplasia have been noted for both active substances. For forchlorfenuron no signs of alpha2u-globulin nephropathy or CPN were noted. For chlorothalonil, CPN was noted in several rat studies. However, as kidney tumours also occurred in mice studies, CPN cannot be the only mode of action behind the tumours. In the list of endpoints for chlorothalonil the mechanistic studies on kidney lesions have been summarised: “The data indicate that kidney lesions are probably caused by thiol metabolites leading to cell-degeneration and subsequent cell-proliferation”. This is in line with Lock and Hard 2004, which places chlorothalonil in the third mode of action (conjugation with GSH) as the nephrotoxic thiol metabolites are formed after metabolism via conjugation with GSH.

For benfluralin the most likely mode of action is number 6 (CPN) as the tumours only occurred in a rat study where CPN also was noted. Neither tubular cytotoxicity nor tubular regeneration was noted in that study. Increased alpha2u-globulin has been measured in one rat study with benfluralin. However, the only alpha2u-globulin nephropathy lesion noted was hyaline droplets and in the study with tumours the hyaline droplets were also noted in female rats. It cannot be concluded that the mode of action of tumour-formation is through CPN as the histopathology in the DAR was not described to an extent making it possible to decide whether benfluralin fulfil the criteria for that mode of action.

Thus for two of the active substances inducing tubular neoplasms (chlorotoluron and molinate) the mode of action seem to be pure speculation that do not fit with the available data. For chlorothalonil the mode of action seems to be number 3 (conjugation with GSH followed by cytotoxicity). For forchlorfenuron the mode of action seems to be number 4 (cytotoxicity). For benfluralin the mode of action seems to be number 6 (CPN) although it is not known whether benfluralin meet the criteria for this mode of action.

Mode of action for tubular effects / increased kidney weight:

For mesotrione and sulcotrione, it has been shown that corneal lesions can be attributed to increased plasma tyrosine levels following inhibition of HPPD, a key enzyme of the tyrosine

catabolic pathway. For mesotrione increased kidney weight correlated with plasma tyrosine levels and showed similar dose-response curves. It was not mentioned whether the other effects (hyaline droplets, CPN) correlated with the plasma tyrosin level. However for sulcotrione the effects on the kidney (increased weight, tubular hypertrophy / hyperplasia, papillary degeneration / death, CPN) did not correlate well with either blood or tissue tyrosine concentrations and were considered to be a direct consequence of sulcotrione exposure. Thus it seems that the tyrosine level is not part of the mode of action for kidney effects.

Mode of action for tubular cell degeneration / cell death:

For copper sulphate (copper compounds) it has been shown that the copper accumulated in hyaline droplets in renal tubule epithelium. Accumulation eventually caused severe necrosis followed by regeneration and recovery.

Mode of action for tubular hypertrophy / hyperplasia:

2,4-D has been included both in CAG level 2a: Tubular degeneration / death and CAG level 2c: Tubular hypertrophy / hyperplasia even though it is probably the same lesion that there is referred to. The authors of an article in the open literature are stating that the lesion described in another article (and in the DAR) as degeneration is morphologically similar to the lesion (few scattered foci of tubules with prominenet basophilia due to high nuclear density and decreased cytoplasmic volume of the epithelial cells) described in this paper as simple hyperplasia. The authors are interpreting the renal lesions to be the result of peroxisome proliferator activity although no increase in peroxisome number was detected in the affected tubules. In neighbouring cells to the hyperplastic cells increased activity of P450 4A and catalase (markers of peroxisome proliferation) was detected. The authors are speculating that the affected hyperplastic cells proliferated following a cytotoxic effect possibly related to peroxisome proliferation.

Mode of action for hypertrophy of collecting ducts:

For florasulam hypertrophy of the collecting ducts was reported in most short and long term studies. It is probably the type A intercalated cells that are hypertrophied in the collecting duct. The type A intercalated cells are involved in acid secretion into urine and HCO3 resorption. Hypertrophy of type A intercalated cells has been reported as a physiological response to several factors affecting acid-base homeostasis. Other potential mechanisms of type A intercalated cell hypertrophy include hypokalaemia, altered levels of adrenal mineralocorticoids, carbonic anhydrase inhibition and HCO3/Cl exchange in the basolateral membrane. Florasulam may also have acted directly upon the type A intercalated cell by some unknown mechanism to cause the hypertrophy.

Mode of action for pelvic hyperplasia:

Many mechanistic studies have been conducted with 2-phenylphenol because of the tumours it produces in the urinary bladder. Despite the many studies the mode of action of urinary

bladder toxicity (and thus also pelvic hyperplasia) is not clear-cut. Calculi were produced in a couple of lifetime studies. However, according to the DAR, the mechanism of tumourigenesis in rats was assumed to be non-genotoxic, probably based on chronic irritation of the epithelium by a combination of high pH, high sodium-ion concentration and/or high concentration of free metabolites at high doses. In the studies there was e.g. a positive correlation between urinary pH and the incidence of hyperplasia of the urinary bladder. The tumourigenic potential of 2-phenylphenol was enhanced by co-administration of sodium bicarbonate as an alkalinising agent while the tumourigenesis by its sodium salt was attenuated by co-administration of ammonium chloride as an acidifier.

16.2.4. CAG level 4: Mechanism of action

For a few active substances affecting the kidney, information on the mechanism of action has been proposed. For the remaining substances, no information regarding mechanism of action has been found and consequently, these substances cannot be allocated to a CAG level 4.

16.2.4.1. CAG level 4m1a: Increased calcium in urine

Both fluoxastrobin and fosetyl induce calculi in the kidney and urinary bladder. For both substances mechanistic studies have shown an increased level of phosphorous in the faeces, a decreased level of phosphorous in the urine and an increased level of calcium in the urine. The two substances differed in that the urinary pH was increased for fluoxastrobin but decreased for fosetyl. For fluoxastrobin it was stated that fluoxastrobin resulted in reduced phosphate absorption in the intestine. A potential phosphate deficiency was counter-regulated by reduced renal excretion of phosphate and renal hyper-excretion of calcium. It is proposed by the DAR that increased calcium excretion in urine, together with an increase in urinary pH, led to calculi formation and following erosive and/or irritative effects of these foreign bodies in the urine, moderate to marked diffuse hyperplasia of the transitional epithelium of the urinary tract with inflammation developed.

For 6 other of the active substances (2-phenylphenol, chloridazon, clothianidin, linuron, oxasulfuron, and sulfosulfuron), which induced calculi, mineralisation was noted in the kidney or urinary bladder. Mineralisation in the rat kidney mainly represents calcium salt deposition (Hard et al. 1999). Thus for these substances an increased level of calcium in the urine may also be part of the cause of bladder calculi. However, no mechanistic studies have been performed to confirm or reject that hypothesis.

The active substances identified in mechanistic studies as inducing increased calcium in urine are allocated to CAG level 4m1a and are listed in Table 16.19.

Table 16.19. CAG level 4m1a: Increased calcium in urine

Fluoxastrobin	Fosetyl	

16.2.4.2. Other information reported

Formation of calculi/crystals:

For iprodione the crystals probably consisted of the metabolite 32490 RP. The information on the content of crystals for iprodione is of no relevance for CAGs at level 4 unless other active substances produce the same metabolite and that is not the case. For triasulfuron the calculi mainly contained triasulfuron.

For thiabendazole the main component of calculi was protein. In order to create a CAG at level 4 for thiabendazole, more mechanistic studies are needed to explain whether protein is important for the formation of the calculi.

16.3. Discussion of CAGs for the kidney

One hundred and nineteen active substances were identified to have effects on the kidneys and were allocated to CAG level 1 (Table 16.1). Thirteen distinct CAGs at level 2 have been proposed. Information on mode / mechanism of action is available for only twenty-three of the active substances. The information is summarised in Appendix V.

Ad CAG level 2a, 2b, 2c, and 3n1: Tubular cell degeneration / cell death, fatty changes, and hypertrophy / hyperplasia:

Fifty active substances induce tubular degeneration / death. Thirty-three active substances induce tubular hypertrophy / hyperplasia. Tubular hypertrophy / hyperplasia may develop after tubular necrosis as a compensatory response to loss of tubule function. Actually for 25 out of the 33 active substances, which induce tubular hypertrophy / hyperplasia, tubular degeneration / death has also been noted. For these 25 active substances the tubular hypertrophy / hyperplasia is probably secondary to the tubular necrosis. As explained in the chapter on CAG level 2c, the CAG for tubular hypertrophy / hyperplasia may include some active substances where it is not regenerating tubules that have been noted in the DARs. For 3 out of the 8 active substances that did not induce tubular degeneration / death, CPN was noted. For these 3 active substances it may have been an early stage of CPN that was seen.

Seventeen active substances induce fatty changes as defined in CAG level 2b. This CAG level covers effects described as fatty changes and/or vacuolation. As the vacuoles may or may not contain accumulated fat it may not be all the active substances in this category that actually accumulate fat. Vacuolation may be a transient physiological response or it may represent the initial stage preceding cell degeneration. For twelve out of the seventeen active substances, which induce tubular fatty changes, tubular degeneration / death has also been noted. Thus the fatty changes for these 12 active substances could be considered covered by the CAG for tubular degeneration / death.

For CRA for effects on the kidney it is recommended to consider CAG level 2a: Tubular degeneration / death. It is not recommended to consider CAG level 2c for CRA as the hypertrophy or hyperplasia for most of the substances probably is secondary to the tubular necrosis. Neither is it recommended to consider CAG level 2b as the fatty changes for most of the active substances could be considered covered by CAG level 2a. As oxidative stress (CAG

level 3n1) only have been studied for 2-phenylphenol it is not possible to use CAG level 3n1 for CRA.

Ad CAG level 2d:Tubular neoplasms:

Five substances induce tubular neoplasms. As the mode of action(s) for the tumour formation seem to be different for the five substances it is not recommended to consider CAG level 2d for CRA.

Ad CAG level 2e, 2g, and 3g1: Tubular hyaline droplets and alpha2u-globulin nephropathy:

The 11 active substances in CAG level 2g for alpha2u-globulin nephropathy have in common that they only in male rats induce hyaline droplets and sometimes one or more of the other characteristic lesions of the syndrome. For the 5 active substances in CAG level 3g1, an increased level of alpha2u-globulin has been measured. Thus the two different CAG levels provide different levels of support for the hypothesis that these active substances may induce alpha2u-globulin nephropathy. As alpha2u-globulin nephropathy is not considered relevant to humans, it is not recommended to consider CAG level 2g and 3g1 for CRA.

Five active substances induce hyaline droplets (CAG level 2e) in other species than the rat or also in the female rat. Hyaline droplets are lysosomes containing protein. For one of these active substances, copper sulphate, it has been shown that the copper accumulated in the hyaline droplets. The accumulation eventually caused severe necrosis followed by regeneration and recovery. None of the other active substances in this CAG level induced tubular necrosis but three of them induced CPN. As there are only 5 active substances in this CAG level and the relevance of hyaline droplets in relation to other tubular effects is not known, it is not recommended to consider CAG level 2e for CRA.

Ad CAG level 2f: CPN:

As chemically induced exacerbation of CPN is not considered relevant to humans it is not recommended to consider CAG level 2f for CRA.

Ad CAG level 2h and 2i: Glomerular degeneration / death and inflammation:

Nine active substances induce glomerular degeneration / death and two glomerular inflammation. As discussed in the chapter on CAG level 2h, the effect may represent CPN for two of the active substances included in the CAG. For all but one of the active substances the glomerular effects have only been noted in one study. For all but two of the active substances the glomerular effects are seen together with tubular effects and thus may be secondary to the tubular effects.

As glomerular effects have been noted for very few active substances and it is likely that the glomerular effects are secondary to tubular effects at least for some of the active substances it is not recommended to consider CAG level 2h and 2i for CRA.

Ad CAG level 2j: Inflammation:

Tubular or papillary necrosis may cause inflammation. Calculi in the pelvis may also be the cause of an inflammation in the kidney. Calculi, tubular necrosis or papillary necrosis have been noted for almost all of the 28 active substances, which induce inflammation. As the inflammation probably is secondary to effects already covered in other CAGs for most of the active substances it is not recommended to consider CAG level 2j for CRA.

Ad CAG level 2k and 2l: Papillary degeneration / death and hypertrophy / hyperplasia:

Fourteen active substances induce papillary degeneration / death. The necrotic papilla may undergo regeneration, which may result in papillary hyperplasia. Nine active substances induce papillary hypertrophy / hyperplasia. Five of the active substances that induce papillary hypertrophy / hyperplasia also induce papillary degeneration / death. Thus for these five substances the hyperplasia is probably secondary to the papillary necrosis. Six of the active substances that induce papillary hypertrophy / hyperplasia also induce pelvic hyperplasia. It makes one speculate whether the cause of the papillary hyperplasia at times may be related to the cause of the pelvic hyperplasia.

For CRA for effects on the kidney it is recommended to consider CAG level 2k: Papillary degeneration / death. It is not recommended to consider CAG level 2l for CRA as the hyperplasia for more than half of the substances probably is secondary to the papillary necrosis.

Ad CAG level 2m, 3m1, 3m2, and 4m1a: Pelvic hyperplasia:

Thirty active substances induce pelvic hyperplasia. Sixteen of those also induce hyperplasia in the bladder. Pelvic hyperplasia may be a response to epithelial irritation, which in turn can be induced by urinary crystals, calculi or toxic chemicals. For nineteen of the active substances the epithelial irritation may be explained by urinary crystals (CAG level 3m2) and/or calculi (CAG level 3m1). As calculi frequently are passed during the course of an experiment by the animals without being detected by the investigators, more active substances than those listed may cause pelvic hyperplasia by a calculi mode of action.

For one of the active substances, 2-phenylphenol, where calculi have been found in a couple of studies, calculi does not seem to be considered part of the mode of action for 2-phenylphenol by the DAR. For most of the active substances, mechanistic studies have not been performed, and the DAR has not elaborated on the mode or mechanism of action. For fluoxastrobin and fosetyl mechanistic studies have shown an increased level of calcium in the urine (CAG level 4m1a), which may explain the formation of calculi and the subsequent urinary bladder and pelvic toxicity. For benfluralin the DAR suggests that the calculi is the cause of the pelvic hyperplasia. Thus the DARs have no opinion on whether the calculi or crystals may be the cause of pelvic hyperplasia for 15 out of the 19 active substances that induce calculi or crystals. For 3 substances (fluoxastrobin, fosetyl, and benfluralin) the DAR consider the calculi the cause of the urinary bladder toxicity. And for 2-phenylphenol calculi does not seem to be considered part of the mode of action by the DAR.

For CRA for effects on the kidney it is recommended to consider CAG level 2m: Pelvic hyperplasia. The CAG level 2m may be subdivided into other CAGs at level 3 and 4 as explained in the text above. However, as found calculi and/or crystals not necessarily are the cause of the pelvic hyperplasia it may be wiser to stick to CAG level 2m.

16.4. Recommended CAGs for the kidney

The following CAGs at level 2 are recommended for CRA for effects on the kidney:

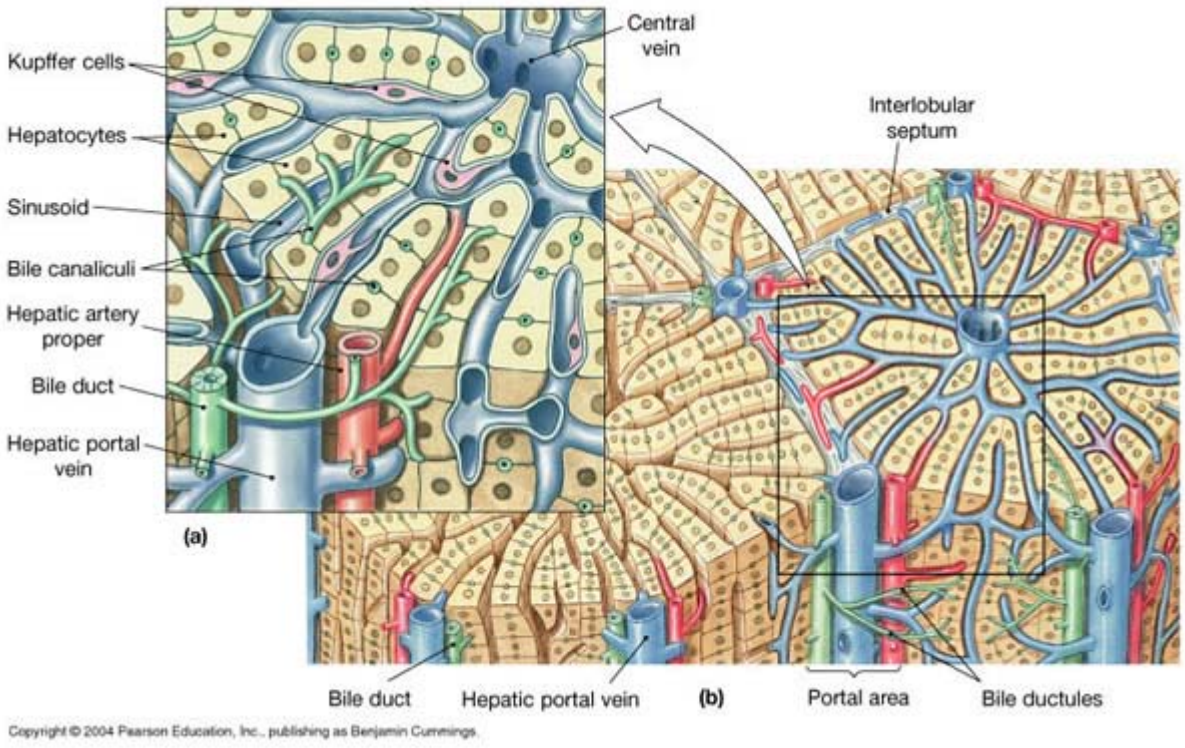
- CAG level 2a: Tubular degeneration / death, see Table 16.2.
- CAG level 2k: Papillary degeneration / death, see Table 16.12.
- CAG level 2m: Pelvic hyperplasia, see Table 16.14.

17. Liver

17.1. Introduction

The liver is the largest organ in the body. It is divided into lobes. Within the liver lobes are multiple, smaller anatomical units (hexagonal structures) called liver lobules, see Figure 17.1.

The liver has a dual blood supply from the hepatic portal vein and from the hepatic artery. Branches of the hepatic portal vein and the hepatic artery are located in the corners of the hexagonal structure in the liver lobules. The blood flows through the sinusoids (small capillaries with highly permeable endothelium) to a central vein in the middle of each liver lobule. The highly permeable endothelium enhances the transport of nutrients and foreign compounds (xenobiotics) into the hepatocytes, which are located in between the sinusoids. The sinusoids are lined with phagocytic cells, Kupffer cells, which remove bacteria and foreign particles from the blood. One of the functions of hepatocytes is to produce bile. The bile flows through the bile canaliculi to bile ducts located in the portal triad together with the branches of the hepatic portal vein and the hepatic artery.



From: http://www.harford.edu/faculty/wrappazzo/BIO204/lecture/bio204lecturematerials.htm

Figure 17.1. Anatomy of the liver lobules.

Functionally, periportal hepatocytes are specialized for oxidative liver functions such as gluconeogenesis, β-oxidation of fatty acids and cholesterol synthesis, while centrilobular hepatocytes are more important for glycolysis, lipogenesis and cytochrome P-450-based drug metabolism (Thoolen et al. 2010).

The liver metabolizes (and stores) a variety of nutrients, endogenous compounds and foreign substances (xenobiotics) (McCance and Huether 1998). Some examples include:

- *Metabolism of carbohydrates*. The liver contributes to the stability of blood glucose levels by releasing glucose during states of low blood sugar and taking up glucose during states of high blood sugar. When glucose is taken up it is stored as glycogen (glycogenesis) or converted to fat. Glucose is released by catabolism of glycogen (glycogenolysis) or by synthesis from amino acids or glycerol (gluconeogenesis).
- *Metabolism of proteins*. Ammonia is released when amino acids are converted to carbohydrates. The liver converts ammonia to urea, which is excreted by the kidneys. The liver synthesizes the plasma carrier protein albumin and globulins (except gamma-globulin). The liver synthesizes most of the coagulation factors, which are necessary for effective blood clotting. The liver also synthesizes a number of enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydogenase (LDH), and alkaline phosphatase (ALP).

- *Metabolism of lipids*. Triglycerides are synthesized from carbohydrates and protein in the liver (lipogenesis). Absorbed lipids enter the liver primarily as triglycerides. The triglycerides may be hydrolyzed and used to produce metabolic energy, or they may be released into the bloodstream as lipoproteins and carried to adipose cells for storage. The liver synthesizes phospholipids and cholesterol, which are needed e.g. for the hepatic production of bile acids, steroid hormones and components of plasma membranes.
- *Production of bile*. Bile is necessary for the digestion of lipids in the intestine. The bile is produced in the liver, stored in the gallbladder, and released into the intestine during digestion of food. Bile consists of bile salts, cholesterol, phospholipids, bilirubin, electrolytes, and water. Bilirubin is a byproduct of destruction of (aged) red blood cells. In the plasma bilirubin binds to albumin (free bilirubin). In the liver the free bilirubin is conjugated to make it a water soluble substance that can be excreted in the bile. Bilirubin gives bile a yellowish green colour. In the intestine the conjugated bilirubin is deconjugated by bacteria and converted to urobilinogen. Some of the urobilinogen is excreted in feces giving it a brown colour. Some of the urobilinogen is reabsorbed and excreted in urine.
- *Storage of minerals and vitamins*. In times of excessive intake the liver stores the vitamins A, B12, D, E and K, and the minerals iron and copper.
- *Metabolism of xenobiotics* (Liska 1998). The liver contains complex enzyme systems for the metabolism of drugs, chemicals in food and environment, and endogenous molecules such as hormones. The purpose of the metabolism of xenobiotics is to create water-soluble compounds that can be excreted in urine. The metabolism often consists of two phases – see Figure 17.2. Phase II reactions are conjugations where a water-soluble molecule such as glutathione, glucuronic acid, or sulphuric acid is added to the molecule. However, many molecules do not have a reactive site that can bind to the water-soluble group. The purpose of phase I reactions are to create metabolites that contain such reactive sites. The phase I reactions are carried out by different cytochrome P450 enzymes.

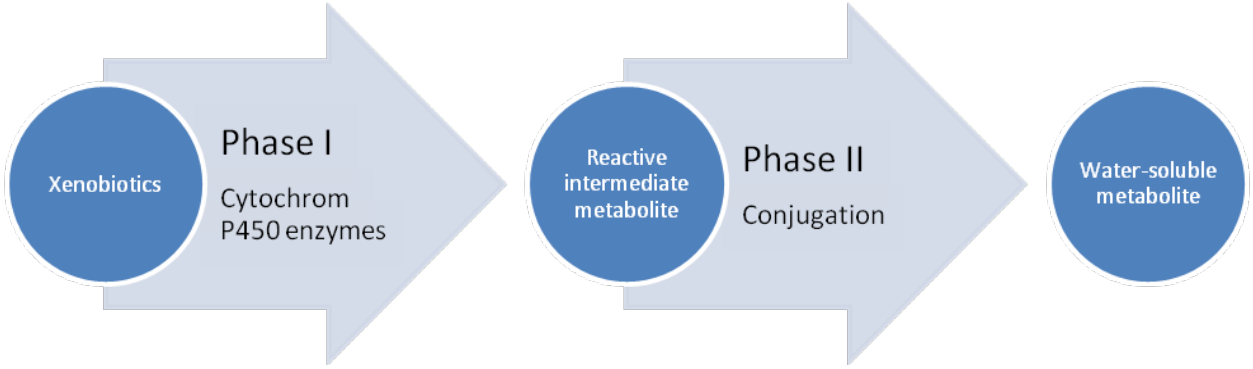


Figure 17.2. Metabolism of xenobiotics

17.2. Establishment of CAGs for toxicity to the liver

17.2.1. CAG level 1: Toxicity to the liver

The active substances identified as having an effect on the liver in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 17.1.

Table 17.1. CAG level 1: Toxicity to the liver

1-Methyl-cyclopropene	Ethofumesate	Molinate
2,4-D	Ethoprophos	Nicosulfuron
2,4-DB	Ethoxysulfuron	Oxadiazon
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Etofenprox	Penconazole
Abamectin (aka avermectin)	Etoxazole	Pendimethalin
Acetamiprid	Famoxadone	Pethoxamid
Acibenzolar-S-methyl (benzothiadiazole)	Fenamidone	Phosmet
Aclonifen	Fenhexamid	Picloram
Alpha-Cypermethrin (aka alphamethrin)	Fenoxaprop-P	Propaquizafop
Amitrole (aminotriazole)	Fenpropidin	Propiconazole
Azimsulfuron	Fenpropimorph	Propineb
Azoxystrobin	Fenpyroximate	Propoxycarbazone
Beflubutamid	Fipronil	Propyzamide
Benalaxyl	Flazasulfuron	Prosulfocarb
Benfluralin	Florasulam	Prosulfuron
Bensulfuron	Fluazinam	Prothioconazole
Bentazone	Fludioxonil	Pymetrozine
Benthiavalicarb	Flufenacet (formerly fluthiamide)	Pyraclostrobin
Benzoic acid	Flumioxazin	Pyraflufen-ethyl
Beta-Cyfluthrin	Fluopicolide	Pyrethrins
Bifenazate	Fluoxastrobin	Pyrimethanil
Bifenox	Flupyrsulfuron-methyl (DPX KE 459)	Pyriproxyfen
Boscalid	Flusilazole	Quinoclamine
Bromoxynil	Flutolanil	Quinoxyfen
Captan	Formetanate	Quizalofop-P-ethyl
Carbendazim	Fuberidazole	Quizalofop-P-tefuryl
Carfentrazone-ethyl	Gibberellin	Rimsulfuron (aka renriduron)
Chloridazon (aka pyrazone)	Glyphosate (incl trimesium aka sulfosate)	Silthiofam
Chlorothalonil	Imazalil (aka enilconazole)	S-Metolachlor
Chlorotoluron	Imazosulfuron	Sodium 5-nitroguaiacolate
Chlorpropham	Imidacloprid	Sodium o-nitrophenolate
Chlorsulfuron	Iodosulfuron-methyl-sodium	Sodium p-nitrophenolate

Cinidon ethyl	Ioxynil	Spinosad
Clodinafob-prop	Iprodione	Spiroxamine
Clofentezine	Iprovalicarb	Sulcotrione
Clomazone	Isoproturon	Tebuconazole
Clopyralid	Isoxaflutole	Tebufenpyrad
Clothianidin	Kresoxim-methyl	Teflubenzuron
Copper compounds	lambda-Cyhalothrin	Tepraloxydim
Cyclanilide	Lenacil	Tetraconazole
Cyflufenamid	Linuron	Thiabendazole
Cyfluthrin	Lufenuron	Thiacloprid
Cyhalofop-butyl	Magnesium phosphide	Thiamethoxam
Cymoxanil	Maleic hydrazide	Thiophanate-methyl
Cypermethrin	Mancozeb	Thiram
Cyprodinil	Maneb	Tolclofos-methyl
Cyromazine	MCPA	Tolylfluanid
Deltamethrin	MCPB	Tralkoxydim
Desmedipham	Mecoprop	Triadimenol
Dicamba	Mecoprop-P	Tri-allate
Dichlorprop-P	Mepanipyrim	Triasulfuron
Difenoconazole	Mesotrione	Tribenuron (aka metometuron)
Diflubenzuron	Metalaxyl-M	Triclopyr
Diflufenican	Metamitron	Trifloxystrobin
Dimethachlor	Metazachlor	Triflusulfuron
Dimethenamid-P	Metconazole	Trinexapac (aka cimetacarb ethyl)
Dimethomorph	Methiocarb (aka mercaptodimethur)	Triticonazole
Dinocap	Methoxyfenozide	Tritosulfuron
Diuron	Metiram	zeta-Cypermethrin
Dodemorph	Metrafenone	Ziram
Epoxiconazole	Metribuzin	Zoxamide
Ethephon	Milbemectin	

17.2.2. CAG level 2: Phenomenological / specific effects on the liver

The hepatocytes have the flexibility to adapt to changing physiological demands with reversible alterations. However, sufficient stress or injurious stimuli may lead to irreversible changes. Often at high doses, targeted cells go through a sequence of cellular degeneration followed by cell death, but at lower doses degenerative changes do not necessarily lead to cell death. Cellular adaptations may lead to increases in cellular organelles and intracellular accumulations of a variety of substances. (Thoolen et al. 2010)

Various types of effects on the liver were identified as a basis for establishing CAGs at level 2. Based on these effects, eleven distinct CAGs at level 2 are proposed. More information is given in Appendix X.

17.2.2.1. CAG level 2a: Hypertrophy

The term hepatocellular hypertrophy is most commonly used to describe the changes in the liver cell following induction of the metabolic enzymes. The increased enzyme activity in the

cell following the induction causes an increase in cell organelles (endoplasmic reticulum, peroxisomes, and mitochondria). Hepatocellular hypertrophy following enzyme induction is considered an adaptive response to chemical stress. However, excessive hypertrophy from enzyme induction may lead to degeneration and cell death. Hepatocellur hypertrophy often is associated with increased liver weight. (Thoolen et al. 2010).

For the purpose of the CAG project, a number of histopathological findings described in the DARs are interpreted to represent hepatocellular hypertrophy and are allocated to a single CAG level 2, termed 'CAG level 2a: Hypertrophy'. See Appendix X for a list of all terms in the DARs interpreted to represent hepatocellular hypertrophy.

The active substances identified as inducing hepatocellular hypertrophy are allocated to CAG level 2a and are listed in Table 17.2.

Table 17.2. CAG level 2a: Hepatocellular hypertrophy

1-Methyl-cyclopropene	Ethoxysulfuron	Penconazole
2,4-D	Etofenprox	Pendimethalin
2,4-DB	Etoxazole	Pethoxamid
Acetamiprid	Famoxadone	Picloram
Aclonifen	Fenamidone	Propaquizafop
Amitrole (aminotriazole)	Fenoxaprop-P	Propiconazole
Azimsulfuron	Fenpropidin	Propyzamide
Azoxystrobin	Fenpropimorph	Prosulfocarb
Beflubutamid	Fenpyroximate	Prosulfuron
Benfluralin	Fipronil	Prothioconazole
Bensulfuron	Flazasulfuron	Pymetrozine
Benthiavalicarb	Fluazinam	Pyraclostrobin
Benzoic acid	Fludioxonil	Pyraflufen-ethyl
Bifenazate	Flufenacet (formerly fluthiamide)	Pyrethrins
Bifenox	Flumioxazin	Pyrimethanil
Boscalid	Fluopicolide	Pyriproxyfen
Bromoxynil	Fluoxastrobin	Quinoxyfen
Captan	Flupyrsulfuron-methyl (DPX KE 459)	Quizalofop-P-ethyl
Carbendazim	Flusilazole	Quizalofop-P-tefuryl
Carfentrazone-ethyl	Flutolanil	Rimsulfuron (aka renriduron)
Chloridazon (aka pyrazone)	Fuberidazole	Silthiofam
Chlorothalonil	Glyphosate (incl trimesium aka sulfosate)	S-Metolachlor
Chlorsulfuron	Imazalil (aka enilconazole)	Spinosad
Cinidon ethyl	Imazosulfuron	Spiroxamine
Clodinafob-prop	Imidacloprid	Sulcotrione
Clofentezine	Iodosulfuron-methyl-sodium	Tebuconazole
Clomazone	Ioxynil	Tebufenpyrad
Clopyralid	Iprodione	Teflubenzuron
Clothianidin	Iprovalicarb	Tepraloxydim
Copper compounds	Isoproturon	Tetraconazole
Cyclanilide	Isoxaflutole	Thiabendazole
Cyflufenamid	Kresoxim-methyl	Thiacloprid
Cyhalofop-butyl	Lenacil	Thiamethoxam

Cymoxanil	Linuron	Thiophanate-methyl
Cypermethrin	Lufenuron	Tolclofos-methyl
Cyprodinil	Mancozeb	Tolylfluanid
Desmedipham	Maneb	Tralkoxydim
Dicamba	Mepanipyrim	Triadimenol
Dichlorprop-P	Mesotrione	Tri-allate
Difenoconazole	Metalaxyl-M	Triasulfuron
Diflubenzuron	Metamitron	Tribenuron (aka metometuron)
Diflufenican	Metazachlor	Triclopyr
Dimethachlor	Metconazole	Trifloxystrobin
Dimethenamid-P	Methoxyfenozide	Triflusulfuron
Dimethomorph	Metiram	Trinexapac (aka cimetacarb ethyl)
Dinocap	Metrafenone	Triticonazole
Diuron	Metribuzin	Tritosulfuron
Dodemorph	Milbemectin	Ziram
Epoxiconazole	Molinate	Zoxamide
Ethofumesate	Oxadiazon	

17.2.2.2. CAG level 2b: Fatty changes

Hepatocellular fatty change is an accumulation of lipid in the hepatocytes because of perturbations in lipid metabolism and disposition. In macrovesicular fatty change the hepatocytes contain a large well-defined single rounded vacuole within each cell. In microvesicular fatty change the hepatocytes are filled with numerous small lipid vacuoles and the affected hepatocytes may have a foamy appearance. In animal studies, it is common to see a mixture of macrovesicular and microvesicular fatty change. Macrovesicular fatty change can be regarded as a physiological adaptation whereas microvesicular fatty change is usually indicative of more serious hepatic dysfunction. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings described as fatty change, lipid droplets, fat in hepatocytes, vacuolation, steatosis, foamy hepatocytes, fatty degeneration, and fatty liver metamorphosis are allocated to a single CAG level 2, termed ‘CAG level 2b: Fatty changes’. See Appendix X for a list of all terms in the DARs interpreted to represent hepatocellular fatty change.

The active substances identified as inducing hepatocellular fatty changes are allocated to CAG level 2b and are listed in Table 17.3.

Table 17.3. CAG level 2b: Hepatocellular fatty changes

1-Methyl-cyclopropene	Dodemorph	Metrafenone
Abamectin (aka avermectin)	Epoxiconazole	Oxadiazon
Acetamiprid	Ethofumesate	Pethoxamid
Alpha-Cypermethrin (aka alphamethrin)	Ethoprophos	Phosmet
Amitrole (aminotriazole)	Ethoxysulfuron	Picloram
Benalaxyl	Etoxazole	Propaquizafop

Benfluralin	Famoxadone	Propiconazole
Bensulfuron	Fenamidone	Prosulfocarb
Bentazone	Fenpropimorph	Prothioconazole
Benthiavalicarb	Fipronil	Pyraflufen-ethyl
Benzoic acid	Florasulam	Pyrethrins
Bifenazate	Fluazinam	Quinoxyfen
Bromoxynil	Flufenacet (formerly fluthiamide)	Quizalofop-P-tefuryl
Captan	Flusilazole	Rimsulfuron (aka renriduron)
Carbendazim	Flutolanil	Silthiofam
Chloridazon (aka pyrazone)	Formetanate	Spinosad
Chlorothalonil	Gibberellin	Spiroxamine
Chlorotoluron	Imazalil (aka enilconazole)	Tebuconazole
Clofentezine	Iodosulfuron-methyl-sodium	Teflubenzuron
Clothianidin	Iprodione	Tepraloxydim
Copper compounds	Iprovalicarb	Tetraconazole
Cyflufenamid	Isoproturon	Thiacloprid
Cymoxanil	Isoxaflutole	Thiamethoxam
Cypermethrin	Lenacil	Tolylfluanid
Deltamethrin	Lufenuron	Tralkoxydim
Desmedipham	Mepanipyrim	Triadimenol
Difenoconazole	Mesotrione	Tri-allate
Diflubenzuron	Metalaxyl-M	Triasulfuron
Diflufenican	Metazachlor	Trifloxystrobin
Dimethenamid-P	Metconazole	Triticonazole
Dimethomorph	Methoxyfenozide	Ziram

17.2.2.3. CAG level 2c: Cell degeneration / cell death

Degeneration of hepatocytes reflects cytoplasmic alterations at the borderline between adaptation with resolution back to normal structure and function, and inability to adapt leading to cell death. It may be difficult to distinguish between hepatocellular hypotrophy, different forms of degeneration, and early necrosis. Degenerated hepatocytes may show increased cytoplasmic granularity, cell swelling, and eosinophilia. Glycogen accumulation and spongiosis hepatis are types of degeneration. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings described as hepatocellular degeneration, hepatocytes with granular cytoplasm, glycogen deposition in liver, and spongiosis hepatis are interpreted as representing degeneration of hepatocytes. See Appendix X for a list of all terms in the DARs interpreted to represent degeneration.

Cell death of the hepatocytes is the ultimate result of irreversible cellular injury. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings described as necrosis and apoptosis are interpreted as representing the same type of effect in the liver. See Appendix X for a list of all terms in the DARs interpreted to represent cell death of hepatocytes.

Very few of the active substances induce degeneration without inducing cell death. Therefore, for the purpose of the CAG project, histopathological findings in the form of degeneration and

cell death in the hepatocytes are allocated to a single CAG level 2, termed 'CAG level 2c: Cell degeneration / cell death'.

The active substances identified as inducing cell degeneration and/or cell death of hepatocytes are allocated to CAG level 2c and are listed in Table 17.4.

Table 17.4. CAG level 2c: Hepatocellular cell degeneration / cell death

2,4-D	Epoxiconazole	Oxadiazon
Acibenzolar-S-methyl (benzothiadiazole)	Ethoprophos	Penconazole
Alpha-Cypermethrin (aka alphamethrin)	Ethoxysulfuron	Pendimethalin
Amitrole (aminotriazole)	Famoxadone	Pethoxamid
Azimsulfuron	Fenhexamid	Phosmet
Azoxystrobin	Fenoxaprop-P	Picloram
Beflubutamid	Fipronil	Propaquizafop
Benfluralin	Flazasulfuron	Propiconazole
Bensulfuron	Florasulam	Propyzamide
Bentazone	Fluazinam	Prosulfocarb
Benthiavalicarb	Fludioxonil	Pymetrozine
Benzoic acid	Flufenacet (formerly fluthiamide)	Pyraclostrobin
Bifenazate	Flumioxazin	Pyraflufen-ethyl
Bifenox	Fluopicolide	Pyriproxyfen
Bromoxynil	Flupyrsulfuron-methyl (DPX KE 459)	Quinoxyfen
Carbendazim	Flusilazole	Quizalofop-P-ethyl
Carfentrazone-ethyl	Flutolanil	Quizalofop-P-tefuryl
Chloridazon (aka pyrazone)	Formetanate	Silthiofam
Chlorothalonil	Fuberidazole	Spinosad
Cinidon ethyl	Gibberellin	Spiroxamine
Clodinafob-prop	Imazalil (aka enilconazole)	Sulcotrione
Clofentezine	Imidacloprid	Tebuconazole
Copper compounds	Iprovalicarb	Teflubenzuron
Cyclanilide	Isoproturon	Tetraconazole
Cyflufenamid	Isoxaflutole	Thiacloprid
Cyhalofop-butyl	Kresoxim-methyl	Thiamethoxam
Cymoxanil	lambda-Cyhalothrin	Thiram
Cypermethrin	Linuron	Tolylfluanid
Cyprodinil	Lufenuron	Tralkoxydim
Cyromazine	Magnesium phosphide	Triadimenol
Deltamethrin	Mancozeb	Tri-allate
Desmedipham	Maneb	Triasulfuron
Dicamba	MCPA	Triclopyr
Dichlorprop-P	MCPB	Trifloxystrobin
Difenoconazole	Mepanipyrim	Triflusulfuron
Diflubenzuron	Metamitron	Triticonazole
Dimethachlor	Metazachlor	Tritosulfuron
Dinocap	Metconazole	Ziram
Diuron	Metrafenone	
Dodemorph	Molinate	

17.2.2.4. CAG level 2d: Inflammation

Infiltration of different inflammatory cells is typically a response to death of hepatocytes. Therefore, inflammatory cell infiltrates may be secondary to the effects already covered in the CAG level 2c for cell degeneration and/or cell death. (Thoolen et al. 2010). However, for about 25% of the active substances where inflammatory cell infiltrates are noted in the DAR this is not accompanied by cell death. Therefore, active substances inducing inflammatory cell infiltrates form their own CAG. As increased activity of Kupffer cells may be seen following inflammatory conditions, increased activity of Kupffer cells is included in this CAG.

For the purpose of the CAG project, histopathological findings described as inflammation, inflammatory foci, liver tissue infiltration, hepatitis, mononuclear cell aggregation, Kupffer cell activity increased, histiocytic hepatic infiltration, reticulo-endothelial system activated, microgranuloma, lymphocyte infiltration, cholangiohepatitis, and cholangitis are allocated to a single CAG level 2, termed 'CAG level 2d: Inflammation'. See Appendix X for a list of all terms in the DARs interpreted to represent inflammation.

The active substances identified as inducing inflammation are allocated to CAG level 2d and are listed in Table 17.5.

Table 17.5. CAG level 2d: Inflammation in the liver

2,4-D	Epoxiconazole	Oxadiazon
2,4-DB	Ethoxysulfuron	Penconazole
Abamectin (aka avermectin)	Etoxazole	Pendimethalin
Acetamiprid	Fenhexamid	Propaquizafop
Acibenzolar-S-methyl (benzothiadiazole)	Fenpropidin	Propiconazole
Alpha-Cypermethrin (aka alphamethrin)	Flazasulfuron	Propyzamide
Azimsulfuron	Florasulam	Pymetrozine
Azoxystrobin	Fluazinam	Pyraflufen-ethyl
Beflubutamid	Fludioxonil	Pyriproxyfen
Benfluralin	Flusilazole	Quinoclamine
Benthiavalicarb	Gibberellin	Quizalofop-P-ethyl
Carbendazim	Imazosulfuron	Quizalofop-P-tefuryl
Carfentrazone-ethyl	Imidacloprid	Silthiofam
Chlorothalonil	Iodosulfuron-methyl-sodium	Sodium 5-nitroguaiacolate
Chlorotoluron	Iprodione	Sodium o-nitrophenolate
Clodinafob-prop	Iprovalicarb	Sodium p-nitrophenolate
Copper compounds	Isoproturon	Spinosad
Cyclanilide	Isoxaflutole	Tebuconazole
Cyflufenamid	lambda-Cyhalothrin	Teflubenzuron
Cyhalofop-butyl	Linuron	Tetraconazole
Cymoxanil	Maleic hydrazide	Thiamethoxam
Cypermethrin	MCPA	Thiram

Cyromazine	MCPB	Tralkoxydim
Deltamethrin	Mepanipyrim	Triadimenol
Desmedipham	Mesotrione	Triasulfuron
Difenoconazole	Metamitron	Triclopyr
Diflubenzuron	Metazachlor	Triticonazole
Dimethachlor	Metconazole	Tritosulfuron
Dimethenamid-P	Metribuzin	Ziram

17.2.2.5. CAG level 2e: Foci of cellular alteration

Foci of hepatocellular alteration are localized increased numbers of hepatocytes. The altered hepatocytes are phenotypically different from surrounding hepatocyte parenchyma. Foci of hepatocellular alteration may be potential precursors of neoplastic transformation but can also be found as non-neoplastic end-stage lesions. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings described as foci of cellular change, cell alteration, eosinophilic foci, and aberrant hepatocytes are allocated to a single CAG level 2, termed 'CAG level 2e: Foci of cellular alteration'. See Appendix X for a list of all terms in the DARs interpreted to represent foci of hepatocellular alteration.

The active substances identified as inducing foci of hepatocellular alteration are allocated to CAG level 2e and are listed in Table 17.6.

Table 17.6. CAG level 2e: Foci of cellular alteration in the liver

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Famoxadone	Propiconazole
Abamectin (aka avermectin)	Fenamidone	Propyzamide
Acibenzolar-S-methyl (benzothiadiazole)	Fenoxaprop-P	Prothioconazole
Benthiavalicarb	Fluazinam	Pymetrozine
Benzoic acid	Fluopicolide	Pyraflufen-ethyl
Boscalid	Flusilazole	Pyrimethanil
Bromoxynil	Fuberidazole	Silthiofam
Carbendazim	Imazalil (aka enilconazole)	S-Metolachlor
Cinidon ethyl	Isoproturon	Tebuconazole
Clodinafob-prop	Isoxaflutole	Teflubenzuron
Clofentezine	Kresoxim-methyl	Tepraloxydim
Clothianidin	Linuron	Tetraconazole
Cyhalofop-butyl	Mancozeb	Thiacloprid
Deltamethrin	Maneb	Thiamethoxam
Dichlorprop-P	Mecoprop-P	Thiram
Diflubenzuron	Metamitron	Tolylfluanid
Dimethomorph	Metconazole	Triadimenol
Dodemorph	Metiram	Tri-allate
Epoxiconazole	Metrafenone	Triclopyr
Ethofumesate	Oxadiazon	Triflusulfuron
Etofenprox	Propaquizafop	

17.2.2.6. CAG level 2f: Neoplasms

For the purpose of the CAG project, histopathological findings in the form of hepatocellular adenoma, hepatocellular carcinoma and hepatoblastoma are allocated to a single CAG level 2, termed ‘CAG level 2f: Neoplasms’.

The active substances identified as inducing hepatocellular neoplasms are allocated to CAG level 2f and are listed in Table 17.7.

Table 17.7. CAG level 2f: Neoplasms

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Imazalil (aka enilconazole)	Propaquizafop
Benfluralin	Ioxynil	Propiconazole
Benthiavalicarb	Iprodione	Propineb
Bromoxynil	Isoproturon	Propyzamide
Carbendazim	Isoxaflutole	Pymetrozine
Cinidon ethyl	Kresoxim-methyl	Pyraflufen-ethyl
Clodinafob-prop	Lenacil	Pyrethrins
Clofentezine	Linuron	Quizalofop-P-tefuryl
Cyflufenamid	Mancozeb	Silthiofam
Difenoconazole	Maneb	Tebuconazole
Dimethachlor	Mecoprop-P	Tebufenpyrad
Dimethenamid-P	Mepanipyrim	Teflubenzuron
Epoxiconazole	Metazachlor	Tepraloxydim
Fenoxaprop-P	Metconazole	Tetraconazole
Fluazinam	Metiram	Thiamethoxam
Fluopicolide	Metrafenone	Thiophanate-methyl
Flupyrsulfuron-methyl (DPX KE 459)	Nicosulfuron	Thiram
Flusilazole	Oxadiazon	Triflusulfuron
Fuberidazole	Pethoxamid	

17.2.2.7. CAG level 2g: Lesions of biliary epithelium

Lesions of biliary epithelium following exposure to the active substances are mainly seen as bile duct hyperplasia. Bile duct hyperplasia is a spontaneous change in portal areas of older animals that may be induced or exacerbated after exposure to chemicals. It may be a consequence of hepatic injury and repair, and obstruction of bile flow. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings described as bile duct hyperplasia, bile duct proliferation, portal triad proliferation and bile duct epithelial hypertrophy are interpreted as representing bile duct hyperplasia. See Appendix X for a list of all terms in the DARs interpreted to represent bile duct hyperplasia.

Cholangiofibrosis, biliary cysts, and bile duct lipid vacuolation are other lesions of biliary epithelium. Cholangiofibrosis is an inflammatory, proliferative, and metaplastic reaction involving bile duct epithelium. The reaction is seen in response to pronounced hepatic parenchymal necrosis. Biliary cysts occur as a dilation of biliary structures and are common in older rats. (Thoolen et al. 2010).

For the purpose of the CAG project, histopathological findings in the form of bile duct hyperplasia, cholangiofibrosis, biliary cysts and bile duct lipid vacuolation are are allocated to a single CAG level 2, termed 'CAG level 2g: Lesions of biliary epithelium'.

The active substances identified as inducing lesions of biliary epithelium are allocated to CAG level 2g and are listed in Table 17.8.

Table 17.8. CAG level 2g: Lesions of biliary epithelium

2,4-DB	Florasulam	Prothioconazole
Azoxystrobin	Fluazinam	Pymetrozine
Beflubutamid	Fludioxonil	Pyraflufen-ethyl
Benthiavalicarb	Flufenacet (formerly fluthiamide)	Pyriproxyfen
Carbendazim	Flumioxazin	Quinoclamine
Cinidon ethyl	Fuberidazole	Quizalofop-P-ethyl
Clodinafob-prop	Iprovalicarb	Silthiofam
Clothianidin	Isoproturon	Sulcotrione
Copper compounds	Kresoxim-methyl	Tebuconazole
Cyclanilide	Magnesium phosphide	Teflubenzuron
Cyflufenamid	MCPA	Tetraconazole
Cyhalofop-butyl	MCPB	Thiabendazole
Desmedipham	Metamitron	Thiamethoxam
Dimethenamid-P	Metazachlor	Thiram
Dodemorph	Metconazole	Tolylfluanid
Epoxiconazole	Metrafenone	Tralkoxydim
Ethephon	Oxadiazon	Trinexapac (aka cimetacarb ethyl)
Ethoprophos	Pendimethalin	Triticonazole
Fenamidone	Propaquizafop	Ziram
Flazasulfuron	Propyzamide	

17.2.2.8. CAG level 2h: Porphyria

In porphyria the active substance interfere with the heme metabolism. The result is deposition of pigments of porphyrin, which is a precursor of heme protein. (Thoolen et al. 2010).

The active substances identified as inducing porphyria are allocated to CAG level 2h and are listed in Table 17.9.

Table 17.9. CAG level 2h: Porphyria

Bifenox	Oxadiazon	Tralkoxydim
Carfentrazone-ethyl	Pyraflufen-ethyl	

17.2.2.9. CAG level 2i: Cholestasis

Cholestasis is decreased bile flow resulting from reduced secretion or from obstruction of the biliary tree. The cause of cholestasis may range from hepatocyte injury leading to defective formation and secretion of bile to interference with bile flow along its excretory pathways. For example, chemicals may selectively block uptake of bile components, interfere with the canalicular excretion of bile, or destroy or distort the membranes, organelles, or ductules responsible for normal bile flow (Mohi-ud-din and Lewis 2004). By obstructing the biliary tree, gallbladder calculi may also be the cause of cholestasis.

Bile pigment is a common finding when there is cholestasis secondary to obstruction of bile flow or when there is perturbation in bile metabolism (Thoolen et al. 2010). Therefore for the purpose of the CAG project, increased bile pigment is interpreted as representing cholestasis.

The active substances identified as inducing cholestasis are allocated to CAG level 2i and are listed in Table 17.10.

Table 17.10. CAG level 2i: Cholestasis

Acibenzolar-S-methyl (benzothiadiazole)	Famoxadone	Quinoxyfen
Bensulfuron	Florasulam	Quizalofop-P-ethyl
Chlorothalonil	Pendimethalin	Quizalofop-P-tefuryl
Clodinafob-prop	Propyzamide	Tepraloxydim
Copper compounds	Prosulfocarb	Triflusulfuron
Desmedipham	Prothioconazole	Triticonazole
Difenoconazole	Pymetrozine	

17.2.2.10. CAG level 2j: Inclusions

Inclusions in the hepatocytes are protrusions of cytoplasm into an invagination of the hepatocyte nuclear membrane (Thoolen et al. 2010).

The active substances identified as inducing inclusions in the hepatocytes are allocated to CAG level 2j and are listed in Table 17.11.

Table 17.11. CAG level 2j: Inclusions in hepatocytes

Beflubutamid	Flupyrsulfuron-methyl (DPX KE 459)	Pethoxamid
Benfluralin	Flusilazole	Propaquizafop
Benthiavalicarb	Imazalil (aka enilconazole)	S-Metolachlor

Cyprodinil	Iprovalicarb	Tetraconazole
Dimethachlor	Mancozeb	Tolylfluanid
Fenpropidin	Maneb	

17.2.2.11. CAG level 2k: Karyocytomegaly

Hepatocellular karyocytomegaly is an increase in the number of diploid nuclei per hepatocyte or an increase in the ploidy level of a single hepatocyte nucleus. Karyocytomegaly may be an age-related phenomenon but can also be induced by xenobiotics. (Thoolen et al. 2010).

The active substances identified as inducing hepatocellular karyocytomegaly are allocated to CAG level 2k and are listed in Table 17.12.

Table 17.12. CAG level 2k: Karycytomegaly

Amitrole (aminotriazole)	Flufenacet (formerly fluthiamide)	Metamitron
Benthiavalicarb	Flupyrsulfuron-methyl (DPX KE 459)	Metazachlor
Carbendazim	Flusilazole	Metrafenone
Carfentrazone-ethyl	Iprovalicarb	Penconazole
Cinidon ethyl	Isoxaflutole	Prothioconazole
Cyflufenamid	Linuron	Silthiofam
Cypermethrin	Mancozeb	Tepraloxydim
Etofenprox	Maneb	Tri-allate
Fenamidone	MCPA	Triticonazole
Fenpropimorph	MCPB	
Fludioxonil	Mepanipyrim	

17.2.2.12. Effects not considered relevant for CAGs at level 2

Changes in blood and urine parameters indicative of liver damage:

Liver related blood and urine parameters can be divided into three groups (Ramaiah 2007):

- Hepatic leakage enzymes
- Cholestatic induction parameters
- Liver function parameters

Hepatic leakage enzymes in blood include alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), ornithine carbamyltransferase (OCT), and sorbitol dehydrogenase (SDH). These enzymes are produced by the liver and will leak out of the membrane into the blood following injury to the liver (due to e.g. hepatitis or necrosis) or alterations in the liver membrane permeability (due to e.g. hepatic glycogen or lipid accumulation).

Cholestatic induction parameters in blood include alkaline phosphatise (ALP), gamma glutamyltransferase (GGT), and bilirubin. The enzymes ALP and GGT show minimal activity in normal hepatic tissue. Following cholestasis the enzyme synthesis increase and they will occur in blood in increased amounts. GGT can also be elevated in cases of bile duct hyperplasia. If the function of the liver is impaired or when biliary drainage is blocked, some of the conjugated bilirubin leaks out of the hepatocytes and appears in the blood and urine. In hemolytic anemia, an increased number of red blood cells are broken down, causing an increase in the amount of free bilirubin in the blood. An increased level of bilirubin in the blood causes the yellow discoloration in jaundice. In standard toxicological studies only total bilirubin (free and conjugated) is measured in blood. Thus it is not possible to distinguish between hemolytic anemia and hepatobiliary injury based on total bilirubin. However, as the free bilirubin is not water soluble, bilirubin will not increase in the urine following hemolytic anemia. The excess free bilirubin will go through all of the normal processing mechanisms in the liver and intestine and will show up as an increase in urine urobilinogen.

Liver function parameters in blood include albumin, coagulation times, urea, bilirubin, triglycerides, and bromosulphophtalein (BSP) retention. Decreased albumin, increased coagulation times, and decreased urea may indicate a decrease in the capacity of the liver to synthesize albumin, coagulation factors and urea (from ammonia). Coagulation times may be measured as activated partial thromboplastin time (APTT) or prothrombin time (PT). Decreases in plasma triglycerides have been noted in several tested hepatotoxic chemicals. BSP is a dye that is injected into study animals. With loss of secretory function of the liver, the dye will be retained in the blood.

Changes in all of the above parameters are more or less specific indicators for damage to the liver. However, the CAGs for different histopathological effects will cover the liver injury indicated by these blood and urine parameters.

Hepatocellular atrophy:

The sizes of the hepatocytes are decreased in hepatocellular atrophy because of too little food intake, hemodynamic changes, or the pressure from neoplasia (Thoolen et al. 2010). Atrophy caused by pressure from neoplasia is secondary to the effect already covered in the CAG level 2f for neoplasms of hepatocytes. Hepatocellular atrophy caused by too little food intake or hemodynamic changes is an indirect effect and therefore not considered relevant in terms of CRA for direct effects on the liver.

Hepatocellular hyperplasia:

Hepatocellular hyperplasia is an increased number of normal hepatocytes. Hepatocellular hyperplasia is often a regenerative response to prior or continuous hepatocellular damage. (Thoolen et al. 2010). Therefore, hepatocellular hyperplasia in almost all cases is secondary to the effects already covered by other CAG´s especially the CAG level 2c for degeneration and/or cell death of hepatocytes.

Fibrosis:

Fibrosis in the liver is the formation of excess connective tissue as a reaction to hepatotoxicity (Thoolen et al. 2010). Therefore, fibrosis in the liver is secondary to the effects already covered by other CAG´s especially the CAG level 2c for degeneration and/or cell death of hepatocytes.

Haemorrhage:

Haemorrhage in the liver is found for very few active substances together with necrosis and inflammation. For these substances hemorrhage in the liver is secondary to the effects already covered in the CAG level 2c for degeneration and/or cell death of hepatocytes or in the CAG level 2d for inflammatory cell infiltrates.

Angiectasis:

Angiectasis is multiple blood-filled cystic spaces in the liver occurring after weakening of sinusoidal walls and/or supporting tissue. Angiectasis is a consequence of perturbations in blood flow and/or drainage e.g. following hepatocellular neoplasms or heart failure. Angiectasis may also be chemically induced. (Thoolen et al. 2010). For most of the active substances inducing angiectasis the effect is probably secondary to the effect already covered in the CAG level 2f for neoplasms of hepatocytes. For the rest of the active substances inducing angiectasis the effect may be either a direct effect or an indirect effect. Indirect effects are not considered relevant in terms of CRA for direct effects on the liver. As it is relatively few active substances that induce angiectasis and it is not possible to distinguish between direct and indirect effects it is not considered relevant to create a CAG for angiectasis.

Lipofuscin pigment:

Lipofuscin pigment represents a breakdown of cell membranes, and is often observed in older animals. Lipofuscin accumulation in the liver may be augmented by certain chemicals. (Thoolen et al. 2010). As it is very few active substances that induce accumulation of lipofuscin pigment, and they all induce other effects on the liver such as cell death it is not considered relevant to create a CAG for lipofuscin pigment.

Extramedullary heamatopoiesis in the liver:

Extramedullary heamatopoiesis in the liver is the production of blood cells in the liver. Extramedullary haematopoiesis is a response to an increased haematopoietic demand for instance following hemolytic anaemia. Both erythroid and granulocytic cells may be present in the aggregates; rarely megakaryocytes may be present. (Thoolen et al. 2010). Extramedullary haematopoiesis is therefore an indirect effect and therefore not considered relevant in terms of CRA for direct effects on the liver.

Haemosiderosis:

Haemosiderosis is accumulation of hemosiderin (precipitated iron) in hepatocytes and Kupffer cells following for instance hemolytic anemia (Thoolen et al. 2010). Haemosiderosis is therefore an indirect effect and therefore not considered relevant in terms of CRA for direct effects on the liver.

17.2.3. CAG level 3: Mode of action

For some of the phenomenological / specific effects on the liver described under CAG level 2, a mode of action has been proposed for several active substances. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

17.2.3.1. CAG level 3a: Phase I enzyme induction

The enzymes involved in the phase I reactions are mainly different cytochrome P450 enzymes. When the body is faced with a high load of an active substance, the phase I and/or phase II enzymes involved in the metabolism of this active substance can be induced, leading to higher amounts of enzymes being present and a higher rate of metabolism. If Phase I metabolism is induced relatively more than Phase II, the level of potential reactive intermediates may increase. The reactive intermediate metabolites may cause toxicities by damaging to proteins, RNA, and DNA (Liska 1998). As an example from humane medicine, the analgesic paracetamol is known to be activated by hepatic CYP2E1 to a reactive metabolite. Chronic administration of ethanol can result in hepatic CYP2E1 induction and hence enhance the hepatotoxicity of paracetamol in humans. (Graham and Lake 2008).

Drugs may lose their desired pharmacological effect when their metabolism is enhanced because of simultaneously exposure to other substances that induce CYPs. Induction of hepatic CYPs in humans may also lead to an increased metabolism of endogenous compounds such as folic acid, steroids steroid hormones, prostaglandins, fatty acids, cholesterol, bile acids,and vitamin D. Thus, induction of hepatic CYPs by xenobiotics may in some cases lead to profound pertubation of endogenous regulatory circuits with associated pathofysiological consequences in other organs and tissues than the liver (Waxman, 1999).

Most CYP forms are induced by receptor-mediated mechanisms leading to an increase in gene transcription. Three nuclear receptor superfamily members, designated CAR (constitutive androstane receptor), PXR (pregnane X receptor), and PPARα (peroxisome proliferator-activated receptor alpha, are mediating induction of hepatic CYP450's belonging to the families CYP2, CYP3, and CYP4 in response to the prototypical inducers phenobarbital (CAR), pregnenolone 16-alpha-carbonitrile and rifampicin (PXR), and clofibrate (PPAR). Induction of CYPs belonging to the family CYP1 is mediated by the AhR (aryl hydrocarbon receptor; Ah receptor) which is member of the PAS transcription family, not a nuclear receptor. The activation of certain receptors also leads to responses that produce effects on genes other than those involved in phase I and II metabolism which in rodent may lead to liver toxicity, including tumours (Waxman, 1999) (see CAG level 4a3 and CAG level 4a6 for further details).

In rodents, the CYP1A, CYP2B, CYP2E, CYP3A and CYP4A families are the main CYPs that may be induced. Induction of hepatic CYPs in rodents may indicate a potential for

induction of CYPs in human liver, although pronounced species differences have been reported in the occurrence of specific CYPs and their induction (Graham and Lake 2008).

Induction of CYP450 species is normally measured as an increased activity of specific biotransformation reactions that are catalysed by the individual CYPs.

Active substances where induction of phase I enzymes has been measured are included in this CAG level. See Appendix X for a list of all terms in the DARs interpreted to represent phase I enzyme induction. In CAG level 4, active substances have been further sub-grouped according to induction of specific CYPs.

The active substances identified as increasing phase I enzymes are allocated to CAG level 3a and are listed in Table 17.13.

Table 17.13. CAG level 3a: Increase in phase I enzymes in the liver

2,4-D	Fluopicolide	Propaquizafop
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Fluoxastrobin	Propiconazole
Alpha-Cypermethrin (aka alphamethrin)	Flupyrsulfuron-methyl (DPX KE 459)	Propoxycarbazone
Benfluralin	Flusilazole	Propyzamide
Benthiavalicarb	Flutolanil	Prothioconazole
Beta-Cyfluthrin	Imazalil (aka enilconazole)	Pymetrozine
Bifenox	Imidacloprid	Pyraflufen-ethyl
Boscalid	Iprodione	Pyrethrins
Chlorpropham	Iprovalicarb	Pyrimethanil
Clodinafob-prop	Isoproturon	Quinoxyfen
Clofentezine	Isoxaflutole	Quizalofop-P-tefuryl
Clothianidin	Kresoxim-methyl	S-Metolachlor
Cyflufenamid	lambda-Cyhalothrin	Spiroxamine
Cyfluthrin	Linuron	Tebuconazole
Cypermethrin	Mancozeb	Tebufenpyrad
Deltamethrin	Maneb	Tetraconazole
Dicamba	MCPA	Thiabendazole
Difenoconazole	Mecoprop	Thiacloprid
Diflubenzuron	Mecoprop-P	Thiamethoxam
Dimethenamid-P	Mepanipyrim	Thiophanate-methyl
Diuron	Mesotrione	Thiram
Epoxiconazole	Metalaxyl-M	Tolclofos-methyl
Ethoprophos	Metconazole	Tralkoxydim
Etofenprox	Metrafenone	Triadimenol
Famoxadone	Metribuzin	Tri-allate
Fenamidone	Oxadiazon	Triflusulfuron
Fenoxaprop-P	Penconazole	zeta-Cypermethrin
Fenpropimorph	Pendimethalin	
Fipronil	Pethoxamid	

The standard toxicological guidelines do not include as a mandatory requirement analysis of the phase I enzymes. Therefore, more active substances than those listed may induce phase I enzymes.

The 'CAG level 2a: Hepatocellular hypertrophy' may be used as an indication for phase I enzyme induction, as the term hepatocellular hypertrophy is most commonly used to describe the changes in the cell following enzyme induction.

17.2.3.2. CAG level 3b: Oxidative stress

Oxidative stress occurs in cells when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability. ROS can be produced in normal cellular metabolism or by inflammatory cells. Reactive intermediate metabolites produced in the metabolism of xenobiotics may enhance the formation of ROS. Antioxidants such as vitamin C, vitamin E, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase normally inactivates ROS. With excessive formation of reactive intermediate metabolites and ROS the antioxidant capacity may be overloaded. The result is oxidative stress which may result in damage to DNA, lipids, and proteins in the cell. Unrepaired DNA damage may lead to mutations and potentially tumours. Oxidative injury may also produce cell death and tumours by the cytotoxic mode of action (see CAG level 3c). (Klaunig et al. 1998).

Active substances which induce oxidative stress measured as increased ROS, free radicals, or malondialdehyde (marker for oxidised lipid) and/or a decreased level of the antioxidants GSH, GSH-Px, SOD or catalase are included in this CAG level.

The active substances identified as inducing oxidative stress are allocated to CAG level 3b and are listed in Table 17.14.

Table 17.14. CAG level 3b: Oxidative stress

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Deltamethrin	Methiocarb (aka mercaptodimethur)
Captan	Gibberellin	Propiconazole
Chlorothalonil	Imazalil (aka enilconazole)	Pyraflufen-ethyl
Cypermethrin	lambda-Cyhalothrin	Thiram

The standard toxicological guidelines do not include as a mandatory requirement studies on oxidative stress. Therefore, more active substances than those listed may cause hepatotoxicity by an oxidative stress mode of action.

17.2.3.3. CAG level 3c: Cytotoxicity

Cytotoxicity is a generally accepted mode of action for development of liver tumours. Continued hepatocyte death can cause compensatory regenerative hyperplasia aimed at maintaining the overall liver mass. Such growth results in more opportunities for

"spontaneous" DNA mutations, allowing mutated cells to accumulate and grow. The mutated cells may give rise to foci of cellular alterations and ultimately liver tumours. Liver tumours formed as a result of sustained cytotoxicity and regenerative hyperplasia are considered relevant for evaluating human cancer risk. (Holsapple et al. 2006).

Active substances which induce degeneration and/or cell death of hepatocytes and foci of cellular alteration and/or liver neoplasms in the same study are included in this CAG level.

The active substances identified as inducing foci of cellular alteration and/or liver neoplasms which may be caused by cytotoxicity are allocated to CAG level 3c and are listed in Table 17.15.

Table 17.15. CAG level 3c: Foci of cellular alteration and/or liver neoplasms which maay be caused by cytotoxicity

Acibenzolar-S-methyl (benzothiadiazole)	Famoxadone	Metconazole
Benfluralin	Fenoxaprop-P	Metrafenone
Benthiavalicarb	Fluopicolide	Oxadiazon
Benzoic acid	Flupyrsulfuron-methyl (DPX KE 459)	Propaquizafop
Bromoxynil	Flusilazole	Propyzamide
Carbendazim	Fuberidazole	Pyraflufen-ethyl
Cinidon ethyl	Imazalil (aka enilconazole)	Silthiofam
Clodinafob-prop	Isoproturon	Tebuconazole
Cyflufenamid	Isoxaflutole	Teflubenzuron
Cyhalofop-butyl	Kresoxim-methyl	Tetraconazole
Dichlorprop-P	Mepanipyrim	Thiamethoxam
Difenoconazole	Metamitron	Triadimenol
Epoxiconazole	Metazachlor	Triflusulfuron

17.2.3.4. CAG level 3d: Hormonal changes

The liver is responsive to sex hormones. Epidemiological studies have shown a small increased risk of hepatocellular adenomas following use of long-term estrogen-containing contraceptives. The key events in liver tumour development in rodents following exposure to estrogenic agents are changes of hormone level or function, altered balance between cell proliferation and apoptosis, and development of foci of cellular alteration. (Holsapple et al. 2006).

Active substances where in vitro studies have shown aromatase induction and/or estrogenicity (see Table 25.78.) and in vivo studies have shown foci of cellular alteration and/or liver tumours are included in this CAG level.

The active substances identified as inducing foci of cellular alteration and/or liver neoplasms which may be caused by hormonal changes are allocated to CAG level 3d and are listed in Table 17.16.

Table 17.16. CAG level 3d: Foci of cellular alteration and/or liver neoplasms which may be caused by hormonal changes

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Dodemorph	Thiacloprid
Clodinafop	Iprodione	Triadimenol
Deltamethrin	Oxadiazon	

The standard toxicological guidelines do not include as a mandatory requirement in vitro studies of aromatase induction and/or estrogenicity. Therefore, more active substances than those listed may cause foci of cellular alteration and/or liver tumours by a hormonal mode of action.

17.2.4. CAG level 4: Mechanism of action

For some active substances, a mechanism of action has been proposed.

17.2.4.1. CAG level 4a1: CYP1A enzyme increased

Induction of the cytochrome P450 enzymes of the CYP1A (CYP1A1 and CYP1A2) family involves activation of the aryl hydrocarbon receptor (AhR), which results in an increase in gene transcription. In addition CYP1A enzymes can be induced by AhR-independent pathways possible involving protein stabilisation. (Graham and Lake 2008, Lake 2009).

The active substances identified as increasing CYP1A enzymes are allocated to CAG level 4a1 and are listed in Table 17.17.

Table 17.17. CAG level 4a1: Increase in CYP1A enzymes

Benthiavalicarb	Imazalil (aka enilconazole)	Quinoxyfen
Clothianidin	Iprodione	S-Metolachlor
Cypermethrin	Isoxaflutole	Spiroxamine
Deltamethrin	Linuron	Tetraconazole
Difenoconazole	Mesotrione	Thiabendazole
Diflubenzuron	Metrafenone	Thiacloprid
Dimethenamid-P	Pethoxamid	Thiamethoxam
Diuron	Propiconazole	Thiophanate-methyl
Etofenprox	Prothioconazole	Tralkoxydim
Fipronil	Pymetrozine	Triadimenol
Fluopicolide	Pyrimethanil	

17.2.4.2. CAG level 4a2: CYP2A enzyme increased

Induction of the cytochrome P450 enzymes of the CYP2A family (CYP2A1 testosterone 7-alpha-hydroxylase) involves activation of the CAR receptor, which results in an increase in gene transcription (Waxman 1999).

Two active substances identified as increasing CYP2A enzymes are allocated to CAG level 4a2 and are listed in Table 17.18.

Table 17.18. CAG level 4a2: Increase in CYP2A enzymes

Diflubenzuron	Propaquizafop	

17.2.4.3. CAG level 4a3: CYP2B enzyme increased

Phenobarbital is the prototype of several rodent hepatocarcinogens that induce tumours by a non-genotoxic mechanism. The mechanism of action involves activation of the nuclear receptor, constitutive androstane receptor (CAR), which results in a pleiotropic response including induction of the cytochrome P450 enzymes of the CYP2B (CYP2B1, CYP2B2), family, increased cell proliferation, inhibition of apoptosis, and liver hypertrophy. Prolonged treatment results in the formation of foci of cellular alteration and subsequently of liver tumours. Phenobarbital is not cytotoxic to the liver cells. In addition to CYP2B enzymes, phenobarbital induces other hepatic CYPs together with phase II enzymes. (Graham and Lake 2008, Holsapple et al. 2006).

Scientists have concluded that rodent CYP2B enzymes inducers would not be expected to produce any increased risk of liver tumours in humans. However, an increased liver size has been noted in humans after prolonged treatment with phenobarbital. (Graham and Lake 2008, Holsapple et al. 2006).

Active substances where induction of CYP2B enzymes has been measured are included in this CAG level. Hepatocellular hypertrophy was noted for most of these substances. Foci of cellular alteration and/or neoplasms of hepatocytes were also noted for most of these substances. Contrary to phenobarbital, many of the substances included in this CAG produce liver cell death, and thus also may be acting through a cytotoxic mode of action.

The active substances identified as increasing CYP2B enzymes are allocated to CAG level 4a3 and are listed in Table 17.19.

Table 17.19. CAG level 4a3: Increase in CYP2B enzymes (phenobarbital-like P450 inducers)

Benthiavalicarb	Iprodione	Pyrethrins
Clothianidin	Isoxaflutole	Pyrimethanil
Cypermethrin	Kresoxim-methyl	S-Metolachlor

Deltamethrin	Mesotrione	Tetraconazole
Difenoconazole	Metconazole	Thiabendazole
Diflubenzuron	Penconazole	Thiacloprid
Dimethenamid-P	Pethoxamid	Thiamethoxam
Fenamidone	Propaquizafop	Thiophanate-methyl
Fipronil	Propiconazole	Triadimenol
Fluopicolide	Prothioconazole	
Imazalil (aka enilconazole)	Pymetrozine	

17.2.4.4. CAG level 4a4: CYP2E enzyme increased

Induction of the cytochrome P450 enzymes of the CYP2E family (CYP2E enzymes are induced by ethanol) involves a posttranslational mechanism by stabilisation of the enzyme mRNA and protein. (Graham and Lake 2008).

Two active substances identified as increasing CYP2E enzymes are allocated to CAG level 4a4 and are listed in Table 17.20.

Table 17.20. CAG level 4a4: Increase in CYP2E enzymes

Deltamethrin	Thiophanate-methyl	

17.2.4.5. CAG level 4a5: CYP3A enzyme increased

Induction of the cytochrome P450 enzymes of the CYP3A family involves activation of the nuclear receptor, pregnane X receptor (PXR), which results in an increase in gene transcription (Graham and Lake 2008, Lake 2009). The prototype rat liver CYPs are CYP3A1, CYP3A2 and CYP3A23 (Waxman 1999).

The active substances identified as increasing CYP3A enzymes are allocated to CAG level 4a5 and are listed in Table 17.21.

Table 17.21. CAG level 4a5: Increase in CYP3A enzymes

2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Etofenprox	Propiconazole
Benthiavalicarb	Fipronil	Propyzamide
Bifenox	Flutolanil	Pymetrozine
Chlorpropham	Imazalil (aka enilconazole)	Pyrethrins
Cyfluthrin	lambda-Cyhalothrin	Tetraconazole
Cypermethrin	Linuron	Thiophanate-methyl
Deltamethrin	Mecoprop	Thiram
Diflubenzuron	Mesotrione	Tolclofos-methyl

Diuron	Metconazole	Tralkoxydim
Epoxiconazole	Pendimethalin	zeta-Cypermethrin
Ethoprophos	Propaquizafop	

17.2.4.6. CAG level 4a6: CYP4A enzymes increased

A wide range of non-genotoxic chemicals have been shown to produce liver enlargement and peroxisome proliferation in rats and mice. Peroxisome proliferation is associated with a marked induction of enzymes of the peroximal fatty acid β-oxidation cycle. The mechanism of action involves activation of the nuclear receptor, peroxisome proliferator activated receptor alpha (PPARα), which results in a pleiotropic response including induction of the cytochrome P450 enzymes of the CYP4A family, and similar key effects as the effects leading to liver tumours in the CYP2B enzymes inducers. The prototype rat liver CYPs are CYP4A1, CYP4A2 and CYP4A3 (Waxman 1999). Peroxisome proliferation may lead to oxidative stress which may contribute to liver tumours by causing indirect DNA damage and/or by contributing to the stimulation of cell proliferation. PPARα agonists also inhibit gap junction intercellular communication and stimulate hepatic Kupffer cells. Both of these effects also may contribute to the induction of cell proliferation (Graham and Lake 2008, Klaunig et al. 2003).

It has been concluded that rodent CYP4A enzymes inducers would not be expected to produce any increased risk of liver tumours in humans. Humans are relatively insensitive or non-responsive to peroxisome proliferation at dose levels that produce a marked response in rodents. However, a reduction in serum lipids, which is the primary therapeutic value of the peroxisome proliferators used as drugs in humans, occurs in all species tested to date. (Graham and Lake 2008, Klaunig et al. 2003).

Active substances where induction of CYP4A enzymes and/or increased activity of palmitoyl CoA oxidase, acyl CoA oxidase, and/or catalase (markers of peroxisome proliferation) have been measured are included in this CAG level.

The active substances identified as increasing CYP4A enzymes are allocated to CAG level 4a6 and are listed in Table 17.22.

Table 17.22. CAG level 4a6: Increase in CYP1A enzymes (peroxisome proliferator activated receptor alpha (PPARα) agonists)

2,4-D	Mecoprop	Pyrethrins
Clodinafob-prop	Mecoprop-P	Quizalofop-P-tefuryl
Dicamba	Oxadiazon	Tebufenpyrad
Famoxadone	Propaquizafop	Tri-allate
Fenoxaprop-P	Propiconazole	
MCPA	Pyraflufen-ethyl	

17.2.4.7. CAG level 4c1: Porphyria and cytotoxicity

In porphyria the active substance interfere with the heme metabolism. The result is deposition of pigments of porphyrin, which is a precursor of heme protein. (Thoolen et al. 2010).

Porphyria may be the cause of persistent hepatocellular injury. Some porphyrinogenic chemicals cause liver tumours by this cytotoxic mode of action. Liver tumours formed as a result of porphyria resulting in sustained cytotoxicity are considered relevant for evaluating human cancer risk. (Holsapple et al. 2006).

Active substances which induce degeneration and/or cell death of hepatocytes and porphyria in the same study are included in this CAG level. Only one of the active substances, Oxadiazon, inducing porphyria induce foci of cellular alteration and/or liver tumours.

The active substances identified as inducing degeneration and/or cell death of hepatocytes and porphyria are allocated to CAG level 4b1 and are listed in Table 17.23.

Table 17.23. CAG level 4c1: Degeneration and/or cell death which may be caused by porphyria

Carfentrazone-ethyl	Oxadiazon	Tralkoxydim

17.3. Discussion of CAGs for the liver

17.3.1. Studies on effects on the liver in experimental animals

The pesticide active substances on the European market typically have been tested in a battery of standard toxicological tests. In the standard guidelines for testing of repeated dose toxicity including chronic toxicological studies in rodents, histopathological changes in the liver such as hepatocellular hypertrophy, fatty change, death, and tumours will be revealed. In addition it is a requirement to measure certain clinical biochemical parameters such as liver enzymes which when increased in the blood indicate liver damage. It is also a requirement to record the liver weight. However, the standard guidelines do not include as a mandatory requirement analysis of the enzymes involved in detoxification in the liver such as the cytochromes P450s. Neither do the standard guidelines require analysis of oxidative stress or hormonal changes that may contribute to liver toxicity. Therefore, for several of the CAG levels more active substances may cause liver toxicity by those mode or mechanisms of action.

17.3.2. Species differences in toxicity to the liver

Although species differences exist, Holsapple et al. 2006 have evaluated that liver tumours caused by a cytotoxic (including porphyria) or hormonal mode of action are relevant for humans. Liver tumours caused by PPAR alpha agonists or phenobarbital-like P450 inducers are not relevant for humans. However, other liver effects may still be relevant for humans exposed to PPAR alpha agonists or phenobarbital-like P450 inducers. It is known that PPAR

alpha agonists lower serum lipids in humans, and phenobarbital may increase the liver size after prolonged treatment in humans (Graham and Lake 2008).

Humans and animals have different hepatic CYPs. However, induction of hepatic CYPs in animals may indicate a potential for induction of CYPs in human liver (Graham and Lake 2008).

17.3.3. Discussion of which CAGs to recommend

One hundred and ninety-five active substances were identified to have effects on the liver and were allocated to CAG level 1. Eleven distinct CAGs at level 2 have been proposed. Information on modes/mechanisms of action is available for a number of the active substances. The information is summarised in Appendix Y.

Foci of cellular alteration and neoplasms of hepatocytes (CAG level 2e and 2f) can be explained by different mode or mechanisms of actions for about 2/3 of the active substances. Cytotoxicity (CAG level 3c), sometimes together with induction of CYP2B enzymes (CAG level 4a3) or CYP4A enzymes (CAG level 4a6), may account for about half of the cases of foci of cellular alteration and/or neoplasms. A possible association between induction of CYP2B enzymes, induction of CYP4A enzymes, or hormonal changes (CAG level 3d) and foci of cellular alteration and/or neoplasms have been found for far less active substances probably because it is not a requirement to study these mode or mechanisms of actions in the standard guidelines. Porphyria may cause neoplasms by a cytotoxic mode of action. However, although porphyria for some of the active substances is associated with cytotoxicity (CAG level 4c), only one of the included active substances which induce porphyria induce neoplasm.

Cell degeneration and/or cell death (CAG level 2c) can be explained by different mode or mechanisms of actions for about 1/3 of the active substances. Phase I enzyme induction (CAG level 3a), sometimes together with oxidative stress (CAG level 3b), may account for about 1/3 of the cases of cell degeneration and/or cell death. A possible association between oxidative stress or porphyria (CAG level 4c) and cell degeneration and/or cell death have been found for very few active substances. Oxidative stress has only been studied for very few active substances as it is not a requirement to study this mode of actions in the standard guidelines. Porphyria is a relatively seldom finding in the studies and thus only may be responsible for cytotoxicity for very few active substances.

Eleven CAG level 2 have been proposed. It should be noted that some of the effects allocated to these CAGs may be interrelated. As the mode or mechanisms of action only may explain a few of the phenomenological effects in the liver for some of the active substances, it is recommended also to consider the eleven CAG level 2 for cumulative risk assessment for effects on the liver.

17.3.4. Chemical classes as basis for CAGs for the liver

Active substances belonging to the same chemical class may have similar toxicological effects. Information in the DARs on effects on the liver is evaluated for similarity of toxicological effects within the relevant chemical classes, i.e. the chemical classes containing more than one active substance. The evaluation includes the active substances allocated to CAG level 1 and the CAG level 2 groups for phenomenological/specific effects. A

comparison of the compounds allocated to CAGs at level 3 and 4 would probably be misleading because only a limited amount of active substances have been studied for mode/mechanism of action.

17.3.4.1. Amides

Both amides, beflubutamid and cyflufenamid, had effects on the liver and induced hypertrophy (CAG level 2a), cell degeneration / cell death (CAG level 2c), inflammation (CAG level 2d), and lesions of biliary epithelium (CAG level 2g). Only cyflufenamid produced fatty changes in the liver (CAG level 2b), liver tumours (CAG level 2f), and karyocytomegaly (CAG level 2k), and only beflubutamid produced inclusions (CAG level 2j). Taken together, the two amides produced a number of similar effects on the liver, but the information in the DARs does not allow a firm conclusion that they as a group always have similar effects on the liver.

17.3.4.2. Anilinopyrimidines

All three anilinopyrimidines, cyprodinil, mepanipyrim, and pyrimethanil, had effects on the liver and induced hypertrophy (CAG level 2a). Cyprodinil and mepanipyrim induced liver cell degeneration / cell death (CAG level 2c). Only mepanipyrim produced fatty changes in the liver (CAG level 2b), inflammation (CAG level 2d), and liver tumours (CAG level 2f) and only cyprodinil induced inclusions (CAG level 2j). Taken together, the three anilinopyrimidines produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that they as a group always have similar effects on the liver.

17.3.4.3. Aryloxyalkanoic acids

All seven aryloxyalkanoic acids (2,4-D, 2,4-DB, dichlorprop-P, MCPA, MCPB, , mecoprop, and mecoprop-P) were identified as having effects on the liver at CAG level 1. However, only 2,4-D, 2,4-DB, and dichlorprop-P induced liver hypertrophy (CAG level 2a), whereas none of the aryloxyalkanoic acids produced fatty changes in the liver (CAG level 2b). 2,4-D, dichlorprop-P, MCPA and MCPB produced liver cell degeneration / cell death (CAG level 2c). 2,4-D, 2,4-DB, MCPA and MCPB produced inflammation (CAG level 2d) whereas only dichlorprop-P and mecoprop-P induced foci of cellular alteration (CAG level 2e) and only mecoprop-P induced liver tumours (CAG level 2f). Lesions of biliary epithelium (CAG level 2g) was seen after 2,4-DB, MCPA and MCPB but none of the substances produced porphyria (CAG level 2h) and cholestasis (CAG level 2i). None of the aryloxyalkanoic acids gave inclusions (CAG level 2j) but MCPA and MCPD induced karyocytomegaly (CAG level 2k), Taken together, the information in the DARs indicate that although all the aryloxyalkanoic acids have effects on the liver, this does not allow the conclusion that the aryloxyalkanoic acids as a group have similar toxic effects on the liver.

17.3.4.4. Aryloxyphenoxypropionates

All five aryloxyphenoxypropionates (clodinafop-P, cyhalofop-butyl, fenoxaprop-P, propaquizafop, and quizalofop-P) were identified as having effects on the liver at CAG level 1. All induced liver hypertrophy (CAG level 2a) but only propaquizafop and quizalofop-P produced fatty changes in the liver (CAG level 2b). All the aryloxyphenoxypropionates

produced liver cell degeneration / cell death (CAG level 2c) and all except fenoxaprop-P produced inflammation (CAG level 2d). Clodinafop-P, cyhalofop-butyl, fenoxaprop-P, and propaquizafop induced foci of cellular alteration (CAG level 2e). Clodinafop-P, fenoxaprop-P, propaquizafop, and quizalofop-P induced liver tumours (CAG level 2f). Lesions of biliary epithelium (CAG level 2g) was seen after clodinafop-P, cyhalofop-butyl, propaquizafop, and quizalofop-P, but none of the substances produced porphyria (CAG level 2h). Cholestasis was induced by clodinafop-P and quizalofop-P (CAG level 2i). Propaquizafop was reported to induce inclusions (CAG level 2j). None of the substances induced karyocytomegaly (CAG level 2k), Taken together, the information in the DARs indicate that all the aryloxyphenoxypropionates have effects on the liver and all produced liver hypertrophy and liver cell degeneration / cell death. However, the information does not allow the conclusion that the aryloxyphenoxypropionates have similar toxic effects on the liver.

17.3.4.5. Benzenamides

All three benzamides (fluopicolide, propyzamide and zoxamide) were identified as having effect on the liver (CAG level 1) and all induced liver hypertrophy (CAG level 2a). Fluopicolide and propyzamide produced liver cell degeneration / cell death (CAG level 2c) and foci of cellular alteration (CAG level 2e). Only propyzamide produced inflammation (CAG level 2d), liver tumours (CAG level 2f), lesions of biliary epithelium (CAG level 2g) and cholestasis (CAG level 2i). Taken together, the information in the DARs indicate that the benzamides have effects on the liver and all produced liver hypertrophy. However, the information does not allow the conclusion that the benzamides as a group have similar toxic effects on the liver.

17.3.4.6. Benzimidazoles

All three benzimidazoles (fuberidazole, thiabendazole, and thiophanate-methyl) were identified as having effect on the liver (CAG level 1) and all induced liver hypertrophy (CAG level 2a), Only fuberidazole produced liver cell degeneration / cell death (CAG level 2c) and foci of cellular alteration (CAG level 2e). Fuberidazole and thiophanate-methyl were reported to induce liver tumours (CAG level 2f). Fuberidazole and thiabendazole produced lesions of biliary epithelium (CAG level 2g). No other effects on the liver were found. Taken together, the information in the DARs indicate that the benzimidazoles have effects on the liver and all produced liver hypertrophy. However, the information does not allow the conclusion that the benzimidazoles as a group have other similar toxic effects on the liver.

17.3.4.7. Benzoic acid and dicamba (aromatic carboxylic acids)

Dicamba classified as a benzoic acid and benzoic acid itself, being classified as an aromatic carboxylic acid, both had effects on the liver (CAG level 1) and both compounds induced liver hypertrophy (CAG level 2a) and liver cell degeneration / cell death (CAG level 2c). Only benzoic acid was reported to produce fatty changes in the liver (CAG level 2b)and foci of cellular alteration (CAG level 2e). None of the compounds produced inflammation (CAG level 2d), liver tumours (CAG level 2f), lesions of biliary epithelium (CAG level 2g), porphyria (CAG level 2h), cholestasis (CAG level 2i), inclusions (CAG level 2j), or

karyocytomegaly (CAG level 2k), Taken together, the information in the DARs indicate that benzoic acid and dicamba have similar toxic effect on the liver.

17.3.4.8. Benzoylureas

All three benzoylureas (diflubenzuron, lufenuron, and teflubenzuron) were identified as having effect on the liver (CAG level 1) and all induced liver hypertrophy (CAG level 2a), fatty changes in the liver (CAG level 2b) and liver cell degeneration / cell death (CAG level 2c). Only diflubenzuron showed inflammation (CAG level 2d) and foci of cellular alteration (CAG level 2e) and only teflubenzuron was reported to induce liver tumours (CAG level 2f). Teflubenzuron also produced lesions of biliary epithelium (CAG level 2g). None of the compounds produced porphyria (CAG level 2h), cholestasis (CAG level 2i), inclusions (CAG level 2j), or karyocytomegaly (CAG level 2k). Taken together, the information in the DARs indicate that the benzoylureas have effects on the liver and all produced liver hypertrophy, fatty changes and liver cell degeneration / cell death. However, the information does not allow a firm conclusion that the benzoylureas have similar toxic effects on the liver.

17.3.4.9. Biscarbamates

Two active substance are biscarbamates (desmedepham and phenmedipham). Desmedipham was identified as having effect on the liver and induced liver hypertrophy (CAG level 2a), fatty changes in the liver (CAG level 2b), liver cell degeneration / cell death (CAG level 2c), inflammation (CAG level 2d), liver tumours (CAG level 2f), lesions of biliary epithelium (CAG level 2g), cholestasis (CAG level 2i) and inclusions (CAG level 2j). None of the compounds were found to induce foci of cellular alteration (CAG level 2e), porphyria (CAG level 2h) and karyocytomegaly (CAG level 2k). Taken together, the information in the DARs does not allow the conclusion that the biscarbamates as a group have similar toxic effects on the liver.

17.3.4.10. Carbamates

Eight active substances are carbamates (carbamate esters or urethanes) (benthiavalicarb, chlorpropham, iprovalicarb, methiocarb, methomyl, oxamyl, pirimicarb, and propamocarb). Benthiavalicarb, chlorpropham, iprovalicarb and methiocarb were reported to have effect on the liver (CAG level 1). Benthiavalicarb and iprovalicarb induced liver hypertrophy (CAG level 2a), fatty changes in the liver (CAG level 2b), liver cell degeneration / cell death (CAG level 2c), inflammation (CAG level 2d), lesions of biliary epithelium (CAG level 2g), inclusions (CAG level 2j and karyocytomegaly (CAG level 2k). Only benthiavalicarb induced foci of cellular alteration (CAG level 2e) and liver tumours (CAG level 2f), None of the compounds induced cholestasis (CAG level 2i) and porphyria (CAG level 2h). Taken together, the information in the DARs does not allow the conclusion that the carbamates as a group have similar toxic effects on the liver.

17.3.4.11. Carboxamides

Both carboxamides, boscalid and diflufenican, had effects on the liver and induced hypertrophy (CAG level 2a). Diflufenican induced fatty changes in the liver (CAG level 2b) and boscalid gave foci of cellular alteration (CAG level 2e). No other effects were noted.

Taken together, the two carboxamides produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that they as a group have similar effects on the liver.

17.3.4.12. Dicarboximides

All three dicarboximides, cinidon ethyl, flumioxazin, and iprodione, had effects on the liver and induced hypertrophy (CAG level 2a). Cinidon ethyl and flumioxazin induced liver cell degeneration / cell death (CAG level 2c) and lesions of biliary epithelium (CAG level 2g), Cinidon ethyl and iprodione induced liver tumours (CAG level 2f), Only iprodione produced fatty changes in the liver (CAG level 2b) and inflammation (CAG level 2d) and only cinidon ethyl produced foci of cellular alteration (CAG level 2e) and karyocytomegaly (CAG level 2k). Taken together, the three dicarboxamides produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that they as a group have similar effects on the liver.

17.3.4.13. Chloroacetamides

All five chloracetamides (dimethachlor, dimethenamid-P, metazachlor, pethoxamid, S-metolachlor) were reported to have effect on the liver (CAG level 1) and all induced hypertrophy (CAG level 2a), Dimethenamid-P, metazachlor and pethoxamid produced fatty changes in the liver (CAG level 2b). Dimethachlor, metazachlor and pethoxamid induced liver cell degeneration / cell death (CAG level 2c). Dimethachlor, dimethenamid-P and metazachlor produced inflammation (CAG level 2d). S-metolachlor induced foci of cellular alteration (CAG level 2e). Dimethachlor, dimethenamid-P, metazachlor and pethoxamid induced liver tumours (CAG level 2f), Dimethenamid-P and metazachlor produced lesions of biliary epithelium (CAG level 2g), dimethachlor and pethoxamid induced inclusions (CAG level 2j), and metazachlor produced karyocytomegaly (CAG level 2k). Taken together, the five chloracetamides produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that they as a group have similar effects on the liver.

17.3.4.14. Diphenylethers

Both diphenylethers, aclonifen and bifenox, had effects on the liver and induced hypertrophy (CAG level 2a). Bifenox induced cell degeneration / cell death (CAG level 2c) and porphyria (CAG level 2h). No other effects in the liver were noted. Taken together, the two diphenylethers produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that they as a group have similar effects on the liver.

17.3.4.15. Dithiocarbamates

Four of the five dithiocarbamates (mancozeb, maneb, propineb, and thiram) and the dimethyldithiocarbamate ziram were identified as having effects on the liver (CAG level 1). Mancozeb, maneb, and ziram induced liver hypertrophy (CAG level 2a), Only ziram produced fatty changes in the liver (CAG level 2b) and inflammation (CAG level 2d). Mancoseb, maneb and ziram induced liver cell degeneration / cell death (CAG level 2c) and mancozeb, maneb, and thiram induced foci of cellular alteration (CAG level 2e). Liver tumours (CAG level 2f) were induced by mancozeb, maneb and thiram. Only thiram and ziram produced

lesions of biliary epithelium (CAG level 2g), Mancoseb and Maneb induced inclusions (CAG level 2j) and karyocytomegaly (CAG level 2k). None of the compounds were found to induce cholestasis (CAG level 2i) and porphyria (CAG level 2h). Taken together, the information in the DARs does not allow the conclusion that the dithiocarbamates as a group have similar toxic effects on the liver.

17.3.4.16. Hydroxybenzonitrils

Both hydroxybenzonitrils, bromoxynil and ioxynil, had effects on the liver and induced hypertrophy (CAG level 2a) and liver tumours (CAG level 2f). Only bromoxynil produced fatty changes in the liver (CAG level 2b), liver cell degeneration / cell death (CAG level 2c), inflammation (CAG level 2d), and foci of cellular alteration (CAG level 2e). No other effects in the liver were noted. Taken together, the two hydroxybenzonitrils produced hypertrophy and liver tumours, but the information in the DARs does not allow a firm conclusion that they as a group have similar effects on the liver.

17.3.4.17. Imidazoles

Both imidazoles, fenamidone and imazalil (aka enilconazole), had effects on the liver and induced hypertrophy (CAG level 2a), fatty changes in the liver (CAG level 2b), foci of cellular alteration (CAG level 2e). Only imazalil produced liver cell degeneration / cell death (CAG level 2c) and inclusions (CAG level 2j), and only fenamidone produced lesions of biliary epithelium (CAG level 2g) and karyocytomegaly (CAG level 2k). Taken together, the two imidazoles produced hypertrophy, fatty changes in the liver (CAG level 2b) and foci of cellular alteration (CAG level 2e), but the information in the DARs does not allow a firm conclusion that they as a group have similar effects on the liver.

17.3.4.18. Morpholines

All four morpholines (dimethomorph, dodemorph, fenpropimorph and spiroxamine) were identified as having an effect on the liver (CAG level 1). All produced liver hypertrophy (CAG level 2a) and fatty changes in the liver (level 2b CAG), Dodemorph and spiroxamine induced liver cell degeneration / cell death (CAG level 2c). Dimethomorph and dodemorph induced foci of cellular alteration (CAG level 2e). Dodemorph induced lesions of biliary epithelium (CAG level 2g), while fenpropimorph induced karyocytomegaly (CAG level 2k). None of the substances induced inflammation (CAG level 2d), liver tumours (CAG level 2f), cholestasis (CAG level 2i), porphyria (CAG level 2h) or inclusions (CAG level 2j). Taken together, the information in the DARs does not allow the conclusion that the morpholines as a group have similar toxic effects on the liver.

17.3.4.19. Neonicotinoids

All five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid and thiamethoxam) were identified as having effects onthe liver (CAG level 1). All five substances induced liver hypertrophy (CAG level 2a). Acetamiprid, clothianidin, thiacloprid and thiamethoxam produced fatty changes in the liver (level 2b CAG) and imidacloprid thiacloprid and thiamethoxam showed liver cell degeneration / cell death (CAG level 2c). Inflammation (CAG level 2d) was reported after acetamiprid, imidacloprid and

thiamethoxam. Foci of cellular alteration (CAG level 2e) were induced by clothianidin, thiacloprid and thiamethoxam.Only thiamethoxam was reported to induce liver tumours (CAG level 2f). Lesions of biliary epithelium (CAG level 2g) were induced by clothianidin, and thiamethoxam, None of the compounds showed cholestasis (CAG level 2i), porphyria (CAG level 2h), inclusions (CAG level 2j and karyocytomegaly (CAG level 2k). Taken together, all five neonicotinoids showed effects onthe liver. However, the information in the DARs does not allow a firm conclusion that the neonicotinoids as a group have similar toxic effects on the liver.

17.3.4.20. Organophosphates

Of the nine organophosphates (chlorpyrifos, chlorpyrifos-methyl, dimethoate, ethoprophos, fenamiphos (aka phenamiphos), fosthiazate, glufosinate, phosmet, pirimiphos-methyl) two (ethoprophos and phosmet) were identified as having effects on the liver (CAG level 1). Ethoprophos and phosmet induced fatty changes in the liver (CAG level 2b) and liver cell degeneration / cell death (CAG level 2c) and ethoprophos induced lesions of biliary epithelium (CAG level 2g). No other specific effects on the liver were reported. Therefore, the information in the DARs does not allow the conclusion that the organophosphates as a group have similar toxic effects on the liver.

17.3.4.21. Oxidazoles

One oxidazole, oxadiazon, had effects on the liver and induced hypertrophy (CAG level 2a), fatty changes in the liver (level 2b CAG), liver cell degeneration / cell death (CAG level 2c). inflammation (CAG level 2d), foci of cellular alteration (CAG level 2e), liver tumours (CAG level 2f), lesions of biliary epithelium (CAG level 2g), and porphyria (CAG level 2h). The other active substance being a oxidazole, oxadiargyl, had no effects on the liver. Taken together, the information in the DARs does not allow the conclusion that the oxidazoles as a group have similar effects on the liver.

17.3.4.22. Phenylureas

One phenylurea, diuron, had effects on the liver and induced hypertrophy (CAG level 2a) and liver cell degeneration / cell death (CAG level 2c). The other active substance being a phenylurea, forchlorfenuron, had no effects on the liver. Taken together, the information in the DARs does not allow the conclusion that the phenylureas as a group have similar effects on the liver.

17.3.4.23. Phthalimides

One phthalamide, captan, had effects on the liver and induced hypertrophy (CAG level 2a) and fatty changes in the liver (level 2b CAG). The other active substance being a phthalamide, folpet, had no effects on the liver. Taken together, the information in the DARs does not allow the conclusion that the phthalamides as a group have similar effects on the liver.

17.3.4.24. Phenylpyrazoles

Both phenylpyrazoles (fibronil and pyraflufen-ethyl) were identified as having an effect on the liver (CAG level 1). Both induced liver hypertrophy (CAG level 2a), fatty changes in the liver (level 2b CAG) and liver cell degeneration / cell death (CAG level 2c). Only pyraflufen-ethyl produced inflammation (CAG level 2d), foci of cellular alteration (CAG level 2e), liver tumours (CAG level 2f), lesions of biliary epithelium (CAG level 2g) and porphyria (CAG level 2h). No other specific effects were reported on the liver. Therefore, the information in the DARs does not allow the conclusion that the phenylpyrazoles have similar toxic effects on the liver.

17.3.4.25. Pyrazoles

Both pyrazoles (fenpyroximate and tebufenpyrad) were identified as having an effect on the liver (CAG level 1). Both induced liver hypertrophy (CAG level 2a) but only tebufenpyrad induced liver tumours (CAG level 2f), No other specific effects were reported on the liver. The information in the DARs does not allow a firm conclusion that the phenylpyrazoles have similar toxic effects on the liver.

17.3.4.26. Pyrethroids and pyrethrin

For eight (alpha-cypermethrin, beta-cyfluthrin, cyfluthrin, cypermethrin, deltamethrin, , etofenprox, lambda-cyhalothrin and zeta-cypermethrin)of the nine active substances classified as pyrethroids effects on the liver were reported in the DARs. For esfenvalerate no effects on the liver were reported. Esfenvalerate lacks the (vinyl)cyclopropanecarboxylate moity characteristic for the majority of the pyrethroid active substances. Cypermethrin and etofenprox were allocated to CAG level 2a: Hypertrophy. Alpha-cypermethrin, cypermethrin, and deltamethrin induced fatty changes in the liver (CAG level 2b). Alpha-cypermethrin, cypermethrin,deltamethrin, and lambda-cyhalothrin produced liver cell degeneration / cell death (CAG level 2c) and inflammation (CAG level 2d). and deltamethrin produced foci of cellular alteration (CAG level 2e). None of the pyrethroids induced neoplasms (CAG level 2f), lesions of biliary epithelium (CAG level 2g), porphyria (CAG level 2h), Cholestasis) (CAG level 2i), or inclusions (CAG level 2j). However, cypermethrin was found to induce karyocytomegaly (CAG level 2k). Taken together, the information in the DARs do not allow the conclusion that the pyrethroids that are cyclopropanecarboxylate esters as a group have similar toxic effects on the liver.

It is noted that pyrethrins are classified as a biopesticide. However, as the pyrethroids are structural derivatives of the naturally occurring pyrethrins, the latter could be considered together with the pyrethroids. According to the DAR pyrethrins produced hypertrophy (CAG level 2a), fatty changes in the liver (CAG level 2b) and liver tumours (CAG level 2f). In conclusion, the information in the DARs do not allows the conclusion that the pyrethroids and pyrethrins as a group have similar toxic effects on the liver.

17.3.4.27. Pyridazones

One pyridazinone, chloridazon, had effects on the liver and induced hypertrophy (CAG level 2a), fatty changes in the liver (level 2b CAG) and liver cell degeneration / cell death (CAG

level 2c). The other active substance being a pyridazinone, flurtamone, had no effects on the liver. Taken together, the information in the DARs does not allow the conclusion that the pyridazones as a group have similar effects on the liver.

17.3.4.28. Pyridine compounds

Four of six pyridine compounds, clopyralid, picloram, pymetrozine, and triclopyr, were identified as having an effect on the liver (CAG level 1). Fluroxypyr and picolinafen had no effects on the liver. Clopyralid, picloram, pymetrozine, and triclopyr induced hypertrophy (CAG level 2a). Only picloram induced fatty changes in the liver (level 2b CAG). Picloram, pymetrozine, and triclopyr produced liver cell degeneration / cell death (CAG level 2c) and pymetrozine and triclopyr induced inflammation (CAG level 2d) and foci of cellular alteration (CAG level 2e). No other effects on the liver were noted. Taken together, the information in the DARs does not allow the conclusion that the pyridine compounds as a group have similar effects on the liver.

17.3.4.29. Sulfonylureas

Fifteen sulfonylureas (azimsulfuron, bensulfuron, chlorsulfuron, ethoxysulfuron, flazasulfuron, flupyrsulfuron-methyl, imazosulfuron, iodosulfuron-methyl-sodium, nicosulfuron, prosulfuron, rimsulfuron, triflusulfuron, triasulfuron, tribenuron, and tritosulfuron) were identified as having effects on the liver. Mesosulferon, metsulfuron-methyl, oxasulfuron, and sulfosulfuron had no effect on the liver. Azimsulfuron, bensulfuron, chlorsulfuron, ethoxysulfuron, flazasulfuron, flupyrsulfuron-methyl, imazosulfuron, iodosulfuron-methyl-sodium, prosulfuron, rimsulfuron, triflusulfuron, triasulfuron, tribenuron, and tritosulfuron induced hypertrophy (CAG level 2a). Bensulfuron, ethoxysulfuron, iodosulfuron-methyl-sodium, rimsulfuron, and triasulfuron induced fatty changes in the liver (CAG level 2b). Cell degeneration / cell death (CAG level 2c) was induced by azimsulfuron, bensulfuron, ethoxysulfuron, flazasulfuron, flupyrsulfuron-methyl, triflusulfuron, triasulfuron, and tritosulfuron. Inflammation (CAG level 2d) was produced by azimsulfuron, ethoxysulfuron, flazasulfuron, imazosulfuron, iodosulfuron-methyl-sodium, triasulfuron, and tritosulfuron, whereas foci of cellular alteration (CAG level 2e) was only induced by triflusulfuron. Flupyrsulfuron-methyl, nicosulfuron and triflusulfuron induced liver neoplasms (CAG level 2f). Lesions of biliary epithelium (CAG level 2g) were seen after flazasulfuron, porphyria (CAG level 2h), Bensulfuron and triflusulfuron induced cholestasis (CAG level 2i) and flupyrsulfuron-methyl produced inclusions (CAG level 2j), and karyocytomegaly (CAG level 2k). Although 15 of 21 sylfonylureas were reported to affect the liver, the information in the DARs does not allow the conclusion that the sulfonylureas as a group have similar toxic effects on the liver.

17.3.4.30. Strobilurines

Five of seven strobilurines were identified as having an effect on the liver. Azoxystrobin, fluoxastrobin, kresoxim-methyl, pyraclostrobin, and trifloxystrobin all induced hypertrophy (CAG level 2a). For the remaining two strobilurines, dimoxystrobin and picoxystrobin, no effects on the liver were reported in the DARs. Fatty changes in the liver (CAG level 2b) were only seen after trifloxystrobin, while azoxystrobin, kresoxim-methyl, pyraclostrobin, and

trifloxystrobin produced cell degeneration / cell death (CAG level 2c). Only azoxystrobin produced inflammation (CAG level 2d) while kresoxim-methyl induced foci of cellular alteration (CAG level 2e) and neoplasms (CAG level 2f). Lesions of biliary epithelium (CAG level 2g) was seen after azoxystrobin and kresoxim-methyl, None of the compound induced porphyria (CAG level 2h), cholestasis (CAG level 2i), inclusions (CAG level 2j), or karyocytomegaly (CAG level 2k). Taken together, the information in the DARs does not allow the conclusion that the strobilurines as a group have similar toxic effects on the liver.

17.3.4.31. Thiocarbamates

All three thiocarbamates (molinate, prosulfocarb, and tri-allate) were identified as having effects on the liver. All three substances produced hypertrophy (CAG level 2a) and cell degeneration / cell death (CAG level 2c), but only prosulfocarb and tri-allate induced fatty changes in the liver (CAG level 2b) and only tri-allate induced foci of cellular alteration (CAG level 2e). Prosulfocarb gave cholestasis (CAG level 2i) and tri-allate induced karyocytomegaly (CAG level 2k). None of the compounds showed inflammation (CAG level 2d), neoplasms (CAG level 2f), lesions of biliary epithelium (CAG level 2g), porphyria (CAG level 2h), and inclusions (CAG level 2j). Taken together, the information in the DARs does not allow the conclusion that the thiocarbamates as a group have similar toxic effects on the liver.

17.3.4.32. Triazoles

All 11 triazoles (amitrole, difenoconazole, epoxiconazole, flusilazole, metconazole, penconazole, propiconazole, tebuconazole, tetraconazole, triadimenol, and triticonazole) had effects on the liver: They all induced hypertrophy (CAG level 2a) and cell degeneration / cell death (CAG level 2c). All except penconazole were reported to produce fatty changes in the liver (CAG level 2b) and all except amitrol were reported to produce inflammation (CAG level 2d). Epoxiconazole, flusilazole, metconazole, tebuconazole, tetraconazole, and triadimenol were reported to produce foci of cellular alteration (CAG level 2e). Neoplasms (CAG level 2f) were induced by difenoconazole, epoxiconazole, flusilazole, metconazole, propiconazole, tebuconazole, and tetraconazole. Lesions of biliary epithelium (CAG level 2g) were seen after epoxiconazole, metconazole, tebuconazole, tetraconazole,and triticonazole, cholestasis (CAG level 2i) after difenoconazole and triticonazole. Inclusions (CAG level 2j) were reported after flusilazole and tetraconazole while flusilazole, penconazole and triticonazole induced karyocytomegaly (CAG level 2k). None of the compounds induced porphyria (CAG level 2h). Taken together, all 11 triazoles as a group produced hypertrophy (CAG level 2a) and cell degeneration / cell death (CAG level 2c), 10 produced fatty changes in the liver (CAG level 2b) and another 10 produce inflammation (CAG level 2d). However, the information in the DARs does not allow a firm conclusion that the triazoles as a group have similar effects on the liver.

17.3.4.33. Triazinones

Both triazinones, metamitron, triazinone (metribuzin), had effects on the liver and induced hypertrophy (CAG level 2a), inflammation (CAG level 2d). Metamitron produced cell degeneration / cell death (CAG level 2c), foci of cellular alteration (CAG level 2e), lesions of

biliary epithelium (CAG level 2g), and karyocytomegaly (CAG level 2k). No other effects in the liver were noted. Taken together, the two triazinones produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that the triazinones as a group have other similar effects on the liver.

17.3.4.34. Triketones

Both triketones, Mesotrione and sulcotrione, had effects on the liver and induced hypertrophy (CAG level 2a). Mesotrione produced fatty changes in the liver (CAG level 2b) and inflammation (CAG level 2d) while sulcotrione induced cell degeneration / cell death (CAG level 2c), No other effects in the liver were noted. Taken together, the two triketones produced hypertrophy (CAG level 2a), but the information in the DARs does not allow the conclusion that the triketones as a group have other similar effects on the liver.

17.3.4.35. Ureas

Two of three ureas were identified as having an effect on the liver. Isoproturon and linuron induced hypertrophy (CAG level 2a), cell degeneration / cell death (CAG level 2c), inflammation (CAG level 2d), foci of cellular alteration (CAG level 2e), neoplasms (CAG level 2f). For the remaining compound being an urea, chlorotoluron, no effects on the liver were reported in the DARs. Only isoproturom produced fatty changes in the liver (CAG level 2b) and lesions of biliary epithelium (CAG level 2g) whereas linuron induced karyocytomegaly (CAG level 2k). Taken together, the information in the DARs does not allow the conclusion that the ureas as a group have similar toxic effects on the liver.

17.3.4.36. Conclusion: Chemical classes as basis for CAGs for the liver

Based on the analysis whether active substances belonging to the same chemical class may have similar effects on the liver it is concluded that active substances belonging to the same chemical class in general do not have similar effects on the liver.

17.4. Recommended CAGs for the liver

The following CAGs are recommended for CRA for effects on the liver:

- CAG level 2a: Hypertrophy, see Table 17.2.
- CAG level 2b: Fatty changes, see Table 17.3.
- CAG level 2c: Cell degeneration / cell death, see Table 17.4.
- CAG level 2d: Inflammation, see Table 17.5.
- CAG level 2e: Foci of cellular alteration, see Table 17.6.
- CAG level 2f: Neoplasms, see Table 17.7.
- CAG level 2g: Lesions of biliary epithelium: see Table 17.8.
- CAG level 2h: Porphyria, see Table 17.9.
- CAG level 2i: Cholestasis, see Table 17.10.

- CAG level 2j: Inclusions, see Table 17.11.
- CAG level 2k: Karyocytomegaly, see Table 17.12.
- CAG level 3a: Phase I enzyme induction, see Table 17.13.
- CAG level 4a1: CYP1A increased, see Table 17.17.
- CAG level 4a2: CYP2A increased, see Table 17.18.
- CAG level 4a3: CYP2B increased, see Table 17.19.
- CAG level 4a4: CYP2E increased, see Table 17.20.
- CAG level 4a5: CYP3A increased, see Table 17.21.
- CAG level 4a6: CYP4A increased, see Table X.22.
- CAG level 3b: Oxidative stress, see Table 17.14.
- CAG level 3c: Cytotoxicity, see Table 17.15.
- CAG level 4c: Porphyria and cytotoxicity, see Table 17.23.
- CAG level 3d: Hormonal changes, see Table 17.16.

18. Lung

18.1. Introduction

The principal function of the lungs is to transport oxygen from the atmosphere into the bloodstream, and to release carbon dioxide from the bloodstream into the atmosphere.

The trachea in humans divides into the two main bronchi that enter the roots of the lungs. The bronchi continue to divide within the lung, and after multiple divisions, give rise to bronchioles. The bronchial tree continues branching until it reaches the level of terminal bronchioles, which lead to alveolar sacs. Alveolar sacs are made up of clusters of alveoli. The individual alveoli are tightly wrapped in blood vessels and it is here that the gas exchange occurs.

There are three major cell types in the walls of the alveoli:

- Type I epithelial cells that form the structure of the alveoli walls.
- Type II epithelial cells that are responsible for the production and secretion of surfactants. The surfactants consists of a group of phospholipids that reduce the alveolar surface tension thus increasing the capability to exchange gases. Type II cells also repair the endothelium of the alveolus when it becomes damaged.
- Macrophages that destroy foreign material, such as bacteria.

18.2. Establishment of CAGs for toxicity to the lungs

Many active substances were identified to affect the skin. The predominant effects include:

- Increased (relative) weight
- Alveolar histiocytosis / alveolar foam cells
- Inflammation
- Neoplasms
- Vacuoles

Some of these effects are non-specific. Other effects are considered as being non-adverse. For the majority of the substances, the overall study NOAELs and LOAELs are lower than for the specific 'lung NOAEL' and 'lung LOAEL'. Moreover, effects on the lungs were generally observed only in one study for a particular active substance – often a long-term study and therefore, the findings were often considered in the DARs to be age-related – not treatment-related. Thus, the lungs seem not to be primary target organs for the active substances included in this project.

Moreover, it is plausible that the effects reported are caused by local irritant properties of the active substances due to that the active substances in the diet (dust) may come in direct contact with the epithelium in the respiratory tract rather than caused following systemic uptake of the substance.

Overall, CRA for the abovementioned effects on the lungs are not considered relevant. Therefore, the lungs are not considered further for CAGs in this project.

18.3. Recommended CAGs for the lungs

No CAGs for toxicity to the lungs are recommended.

19. Lymph node

19.1. Introduction

The main functions of the lymph nodes are to filter pathogens from the afferent lymph and then to initialise immune reactions. A lymph node is enclosed by a capsule and is comprised of various compartments or microenvironments; the outer cortex with primary and secondary follicles (B-lymphocyte areas), the inner cortex or paracortex (T lymphocyte area) and the medulla, with medullary cords and medullary sinuses (contains both T and B lymphocytes).

Lymphocytes and antigens pass into the lymph node via the afferent lymphatics. The lymph (fluid and lymphocytes) drains through the node and passes out of the medulla through the efferent lymphatic vessel. In addition, lymphocytes can enter the organ by the lymph node blood supply. In lymph nodes, antigen presenting cells (APCs) and reactive lymphocytes are

in close contact in the same microenvironment. Upon antigenic stimulation, proliferating cells in the lymph node (clonal expansion) form lymphoid follicles containing foci (germinal centres) of antibody producing B cells, T cells and dendritic cells. The nature of antigenic stimulus and duration of immune response direct the types and composition of T cell subsets in stimulated lymphoid tissues.

After birth, exposure to many new exogenous antigens promotes lymph nodes to develop quickly. Due to the continuously exposure to antigens via the digestive tract, sinal histiocytosis (considerable numbers of macrophages in the sinuses) and accumulations of pigmented macrophages are often present in the mesenteric lymph nodes.

19.2. Establishment of CAGs for toxicity to the lymph nodes

Many active substances were identified to affect the lymph nodes. The predominant effects reported include:

- Increased (relative) weight
- Hypertrophy / hyperplasia
- Decreased (relative) weight
- Atrophy
- Inflammation
- Haemorrhage
- Congestion

Most of the studies describing effects on lymph nodes are test guideline toxicity studies where haematological parameters such as white blood cells (WBC) and/or lymphoid cells are recorded together with macroscopical and/or microscopical changes. The predominant lymph node related phenomenological effects reported in the DARs are increased/decreased weight and hypertrophy/atrophy. For some of the active substances, such effects were regarded as a critical effect and together with critical effects reported for other organs formed the basis of study NOAELs. But for the majority of the substances, the overall study NOAELs and LOAELs are lower than for the specific ‘lymph node NOAEL’ and ‘lymph node LOAEL’. Moreover, effects on the lymph nodes were generally observed only in one study for a particular active substance – often a long-term study and therefore, the findings were often considered in the DARs to be age-related – not treatment-related. Thus, the lymph nodes seem not to be primary target organs for the active substances included in this project.

Overall, CRA for effects on the lymph nodes are not considered relevant. Therefore, the lymph nodes are not considered further for CAGs in this project.

19.3. Recommended CAGs for the lymph nodes

No CAGs for toxicity to the lymph nodes are recommended.

20. Muscles

20.1. Introduction

The function of muscles is to produce force and cause motion. Muscles can cause either locomotion of the organism itself or movement of organs.

Each muscle fibre is a single muscle cell. The muscle fibres contain bundles of myofibrils which are the functional units of muscle contraction. Myofibrils are composed of two contractile proteins, actin and myosin.

In skeletal muscles, contraction is stimulated by electrical impulses transmitted by the efferent nerves in the peripheral nerve system. All skeletal muscle contractions are facilitated by the neurotransmitter acetylcholine.

20.2. Establishment of CAGs for toxicity to the muscles

20.2.1. CAG level 1: Toxicity to the muscles

The active substances identified as having an effect on the muscles in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 20.1.

Table 20.1. CAG level 1: Toxicity to the muscles

Benfluralin	Mancozeb	Spinosad
Chloridazon (aka pyrazone)	Maneb	Thiacloprid
Dimethachlor	Metiram	Thiram
Fosthiazate	Molinate	Trifloxystrobin
Imazaquin	Pirimicarb	Ziram
Isoxaflutole	Propineb	

20.2.2. CAG level 2: Phenomenological / specific effects on the muscles

Only one type of effect on muscles was identified as the basis for establishing CAGs at level 2. More information is given in Appendix Z.

20.2.2.1. CAG level 2a: Weakness

The active substances included in the CAG level 2a for muscle weakness produced histopathological effects described as muscle atrophy, muscle dystrophy, muscle fibre degeneration, and myopathy. Muscle atrophy is a decrease in the mass of the muscle; muscle dystrophy is progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissue; myopathy is a functional defect of muscle fibres.

The active substances identified as inducing muscle weakness are allocated to CAG level 2a and are listed in Table 20.2.

For some of the substances muscle fibres were replaced by connective tissue (spinosad and probineb) or adipose tissue (metiram and ziram). For other substances the muscles were infiltrated with macrophages (spinosad, benfluralin, and thiacloprid).

Table 20.2. CAG level 2a: Muscle weakness

Benfluralin	Mancozeb	Spinosad
Chloridazon (aka pyrazone)	Maneb	Thiacloprid
Dimethachlor	Metiram	Thiram
Fosthiazate	Molinate	Trifloxystrobin
Imazaquin	Pirimicarb	Ziram
Isoxaflutole	Propineb	

20.2.3. CAG level 3: Mode of action

Two different modes of action (nerve degeneration, and formation of a common intermediate metabolite: CS_2) were identified for some of the active substances allocated to CAG level 2.

20.2.3.1. CAG level 3a1: Nerve degeneration

The muscles are enervated by efferent nerves such as the sciatic nerve. When the nerves enervating the muscles are degenerated, the muscles will atrophy and weaken.

The active substances for which muscle weakness is related to nerve degeneration are allocated to CAG level 3a1 and are listed in Table 20.3.

Both for benfluralin and thiacloprid, the DAR suggests that the sciatic nerve degeneration was attributed to the extremely high toxicity rather than to a specific neuropathy. As thiacloprid is a nicotinergic acetylcholine receptor agonist, the DAR is also suggesting that this may be the cause of the exacerbated normal age-related processes in the nerve and muscle tissues. Like thiacloprid, isoxaflutole is a nicotinergic acetylcholine receptor agonist.

Table 20.3. CAG level 3a1: Muscle weakness related to nerve degeneration

Benfluralin	Molinate	Thiram
Isoxaflutole	Thiacloprid	Ziram
Mancozeb		

20.2.3.2. CAG level 3a2: Formation of carbon disulphide

Inhalation studies with propineb suggest that its muscle toxicity appear to be associated with the intermediary breakdown product carbon disulphide (CS_2).

The active substances (dithiocarbamates) having carbon disulphide as a common intermediate metabolite are allocated to CAG level 3b and are listed in Table 20.4

Table 20.4. CAG level 3a2: Muscle weakness related to formation of carbon disulphide

Mancozeb	Metiram	Thiram
Maneb	Propineb	Ziram

20.2.4. CAG level 4: Mechanism of action

No mechanism of actions were identified to form the basis for CAG level 4 for toxicity to the muscles.

20.3. Discussion of CAGs for the muscles

Seventeen active substances were identified to have effects on the muscles and were allocated to CAG level 1. One distinct CAG at level 2 have been proposed. Information on mode of action is available for a number of the active substances. No information on the mechanism(s) of action is available for any of them. The information is summarised in Appendix AA.

Two different modes of action (CAG level 3a1: Nerve degeneration, and CAG level 3a2: Formation of carbondisulphide) were identified for some of the active substances allocated to CAG level 2.

For seven of the seventeen active substances allocated to CAG level 2a, the available information on mode of action (CAG level 3a1) indicates that the effects are indirect effects in the muscles, i.e., are secondary to effects on the nervous system. The CAG level 3a1 as well as the CAG level 2a for these substances are therefore, not considered relevant in terms of CRA for a direct effect on the muscles.

For six of the seventeen active substances allocated to CAG level 2a, the available information on mode of action (CAG level 3a2) indicates that the effects in the muscles are associated with the intermediary breakdown product carbon disulphide (CS_2). This is considered as being a direct effect of these substances on the muscles and therefore, relevant for CRA.

As no information regarding the mode / mechanism(s) of action for the remaining active substance allocated to CAG level 2a has been found, the CAG level 2a could be considered for CRA for effects on the muscles for these substances.

20.4. Recommended CAGs for the muscles

The following CAGs are recommended for CRA for effects on the muscles:

- CAG level 3a2: Formation of carbon disulphide, see Table 20.4.
- CAG level 2a: Weakness, see Table 20.2 (only if no information on the mode / mechanism of action is available).

21. Nervous system

21.1. Introduction

The nervous system is responsible for the body's ability to interact with the environment and for the regulation and coordination of activities involving internal organs. It is a network composed of complex structures that transmit electric and chemical signals between the brain and the body's many organs and tissues.

Structurally, the nervous system is divided into the central nervous system and the peripheral nervous system.

The central nervous system (CNS) consists of the brain and the spinal cord, enclosed within the protective cranial vault and the vertebral column, respectively.

The three major divisions of the brain are:

- The forebrain (telencephalon), formed by the two cerebral hemispheres
- The midbrain (mesencephalon)
- The hindbrain (metencephalon), which includes the cerebellum, pons, and medulla oblongata.

The midbrain, medulla oblongata, and pons make up the brain stem, which connects the hemispheres of the brain, cerebellum, and spinal cord.

The peripheral nervous system (PNS) is composed of the cranial nerves, which project from the brain and pass through foramina (openings) in the skull, and the spinal nerves, which project from the spinal cord and pass through intervertebral foramina of the vertebrae. Peripheral nerve pathways are differentiated into afferent pathways that carry sensory impulses toward the CNS (sensory division), and efferent pathways that innervate effector organs (organs innervated by specific components of the nervous system), such as skeletal, cardiac, and smooth muscles, as well as glands, by transmitting motor impulses away from the CNS (motor division).

Clinically, the nervous system can be divided into the somatic nervous system and the autonomic nervous system. The somatic nervous system consists of motor and sensory pathways regulating voluntary motor control of skeletal muscles. The autonomic nervous

system also consists of motor and sensory components and is involved in regulation of the body's internal environment (viscera) through involuntary control of organ systems.

The autonomic nervous system is further divided into sympathetic and parasympathetic systems. Many organs are innervated by both the sympathetic and parasympathetic systems and the two systems frequently cause opposite responses.

The two basic types of cells that make up the nervous tissue are neurons and supporting cells, such as neuroglial cells of the CNS and Schwann cells of the PNS.

Neurons are the primary cells of the nervous system and generate and conduct electrical and chemical impulses. Impulses are transmitted across a synapse (the region between adjacent neurons) by chemical conduction. The conducting substance is called a neurotransmitter. More than 30 substances are thought to be neurotransmitters, including norepinephrine, acetylcholine, dopamine, histamine, and serotonin. Several neurotransmitters are amino acids, including gamma-aminobutyric acid (GABA), glutamic acid, and aspartic acid. Many of these transmitters have more than one function.

Neurotoxicity is any adverse effect on the structure or function of the nervous system related to exposure to a chemical substance. Neurotoxicity may be indicated by morphological (structural) changes in the central or peripheral nervous system or in special sense organs, neurophysiological changes (e.g., electroencephalographic changes), behavioural (functional) changes, and/or neurochemical changes (e.g., neurotransmitter levels). Symptoms of neurotoxicity may appear immediately after exposure or be delayed.

They may include limb weakness or numbness, loss of memory, vision, and/or intellect, uncontrollable obsessive and/or compulsive behaviors, delusions, headache, cognitive and behavioral problems.

This section deals with neurotoxicity to both the peripheral and central parts of the nervous systems.

21.2. Establishment of CAGs for toxicity to the nervous system

21.2.1. CAG level 1: Toxicity to the nervous system

The active substances identified as having an effect on the nervous system in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 21.1.

Table 21.1. CAG level 1: Toxicity to nervous system

2,4-D	Ethoprophos	Oxamyl
Abamectin (aka avermectin)	Fenamiphos (aka phenamiphos)	Oxasulfuron
Acetamiprid	Fenpropidin	Phenmedipham
Alpha-Cypermethrin (aka alphamethrin)	Fenpropimorph	Phosmet
Benfluralin	Fipronil	Pirimicarb
Benzoic acid	Florasulam	Pirimiphos-methyl
Beta-Cyfluthrin	Flufenacet (formerly fluthiamide), and metabolite	Propamocarb
Chlormequat (chloride)	Formetanate	Propineb

Chlorpropham	Fosetyl	Prosulfocarb
Chlorpyrifos	Fosthiazate	Pyrethrins
Chlorpyrifos-methyl	Glufosinate	Quinoclamine
Clothianidin	Imidacloprid	Spinosad
Cyfluthrin	Indoxacarb	Sulcotrione
Cymoxanil	Isoxaflutole	Thiacloprid
Cypermethrin	Lufenuron	Thiram
Deltamethrin	Mancozeb	Tolclofos-methyl
Desmedipham	Maneb	Triadimenol
Dicamba	Mepiquat	Tri-allate
Dimethoate	Methiocarb (aka mercaptodimethur)	Triflusulfuron
Dimoxystrobin	Methomyl	Ziram
Dinocap, and metabolites	Metiram	lambda-Cyhalothrin
Esfenvalerate	Molinate	zeta-Cypermethrin
Ethephon		

21.2.2. CAG level 2: Phenomenological / specific effects on the nervous system

Various types of effects on the nervous system were identified as a basis for establishing CAGs at level 2. These effects concern functional changes related to the motor division, effects on reflex action, effects on cognition, hypertrophy / hyperplasia in the brain, cell degeneration / cell death in the brain, and neoplasms in the brain. Based on these effects, three distinct CAGs at level 2 are proposed. More information is given in Appendix AB.

21.2.2.1. CAG level 2a: Functional changes related to the motor division

A number of different functional changes in the motor division of the nervous system have been reported in the toxicological studies with active substances. These changes involve effects on the movement of muscles, effects on locomotion, and neuropathy.

Effects related to the movement of muscles include:

- Effects on motor activity - effects on the activity of a muscle, nerve, or centre that affects or produces movement
- Ataxia - lack of control of muscular coordination / irregularity of muscular action due to defects in the nervous system
- Choreoathetosis - a condition marked by choreic and athetoid movements, i.e. involuntary movements
- Abnormal gait - abnormal walking
- Paralysis - condition where the muscles in a part of the body become weak and cannot be moved because the motor nerves have been damaged
- Neuromuscular dysfunction - abnormal functioning of nerves and muscles

The above-mentioned effects related to the movement of muscles could to some extent be considered as a single group of effects and/or some of the effects could be considered as a subgroup of others. For the purpose of the CAG project, these effects are allocated to a single CAG level 2, termed 'CAG level 2a: Functional changes related to the motor division'.

Locomotion is movement or the ability to move from one place to another. Locomotor activity is pertaining to or affecting the locomotive apparatus of the body. Decreased locomotion could be due to decreased ability to move e.g. because of disturbances in the above mentioned functional changes related to the motor division. But it is also noted that decreased locomotor activity could also be due to effects such as pain, exhaustion, or decreased activity in centres regulating locomotion. However for the purpose of the CAG project, effects on locomotion are allocated to 'CAG level 2a: Functional changes related to the motor division'.

Neuropathy is a general term denoting functional disturbances and/or pathological changes in the peripheral nervous system. Organophosphate-induced delayed neuropathy (OPIDN) is a neuropathy caused by destruction of neurons in the central nervous system, especially in the spinal cord, as a result of acute or chronic organophosphate poisoning. The exact OECD TG 418/419 definition is: "Delayed neurotoxicity is a syndrome associated with prolonged delayed onset of ataxia, distal axonopathies in spinal cord and peripheral nerve, and inhibition and aging of neuropathy target esterase in neural tissue". For the purpose of the CAG project, neuropathy and OPIDN are allocated to the CAG level 2a: 'Functional changes related to the motor division'.

The active substances identified as inducing one or more of the abovementioned effects and allocated to CAG level 2a are listed in Table 21.2.

Table 21.2. CAG level 2a: Functional changes related to the motor division

2,4-D	Fenpropidin	Molinate
Abamectin (aka avermectin)	Fenpropimorph	Oxamyl
Acetamiprid	Fipronil	Oxasulfuron
Alpha-Cypermethrin (aka alphamethrin)	Florasulam	Phosmet
Benfluralin	Flufenacet (formerly fluthiamide), and metabolite	Pirimicarb
Beta-Cyfluthrin	Formetanate	Propineb
Chlormequat (chloride)	Fosetyl	Prosulfocarb
Chlorpropham	Fosthiazate	Pyrethrins
Chlorpyrifos	Glufosinate	Spinosad
Chlorpyrifos-methyl	Imidacloprid	Sulcotrione
Clothianidin	Indoxacarb	Thiacloprid
Cyfluthrin	Isoxaflutole	Thiram
Cypermethrin	Lufenuron	Triadimenol
Deltamethrin	Mancozeb	Tri-allate
Dicamba	Maneb	Ziram
Dinocap, and metabolites	Mepiquat	lambda-Cyhalothrin
Esfenvalerate	Methomyl	zeta-Cypermethrin
Ethephon	Metiram	

21.2.2.2. CAG level 2b: Effects on reflex action

A reflex is defined as an involuntary, almost instantaneous, movement in response to a stimulus. Reflexes are dependent on the sensory (afferent) division of the nervous system to detect and conduct impulses in response to a stimulus, and subsequently the motor (efferent) division to conduct impulses to muscles, so that movement occurs. Substances having effects on reflex action could potentially have effects on the sensory and/or motor division of the nervous system. Thus substances in this CAG could very well overlap with the previous CAG "Functional changes related to the motor division". However for the purpose of the CAG project, a distinct CAG on reflex action was established as substances with effects on reflex action could be acting on the sensory division of the nervous system. In the present CAG substances having effects on the righting reflex and/or on the acoustic startle reflex and/or the patellar reflex were included. The righting reflex is the ability to assume an optimal position when there has been a departure from it. Acoustic startle response latency is the response to a sudden unexpected stimulus such as a loud noise. The patellar reflex helps maintain balance and posture

The active substances identified as inducing effects on reflex action, and allocated to CAG level 2b are listed in Table 21.3.

Table 21.3. CAG level 2b: Effects on reflex action

2,4-D	Fenpropimorph	Molinate
Abamectin (aka avermectin)	Fipronil	Phosmet
Clothianidin	Florasulam	Thiram
Cyfluthrin	Flufenacet	Triadimenol
Deltamethrin	Mepiquat	zeta-Cypermethrin
Esfenvalerate		

21.2.2.3. CAG level 2c: Effects on cognition

Cognitive function is an intellectual process by which one becomes aware of, perceives, or comprehends ideas. It involves all aspects of perception, thinking, reasoning, and remembering. It should be noted that only few investigations on learning and memory performance have been performed with the active substances included in this project. Thus, this group is potentially larger than described here

The active substances identified as inducing effects on cognition and allocated to CAG level 2c are listed in Table 21.4.

Table 21.4. CAG level 2c: Effects on cognition

Beta-Cyfluthrin	Clothianidin	Maneb
Chlorpropham	Fipronil	Molinate
Chlorpyrifos		

21.2.2.4. Effects not considered relevant for CAGs at level 2

The presence of vacuoles in the brain is considered as being a non-adverse effect and therefore, not relevant for CAGs at level 2 and consequently, not relevant in terms of CRA for effects on the nervous system.

Many active substances were reported to increase or decrease the relative weight of the brain. In general, the changes were small in comparison to the control group, did not reach statistically significance, were not dose-related, increased weights were related to an increased incidence of masses (noted in the macroscopic examination) and/or neoplasms (noted in the microscopic examination), observed in only one or a few studies, and/or findings were not consistent across studies, sex and/or species. Therefore, the changes in relative brain weight were often considered in the DARs not to be treatment-related. Moreover, the description in the various DARs are very different regarding details and exactness. In conclusion, increased and decreased relative brain weight for the active substances included in Annex I of Council Directive 91/414/EEC (up to 31st of May 2009) are considered as not being applicable for a CAG at level 2 and consequently, not relevant in terms of CRA for effects on the nervous system.

Several active substances were evaluated for induction of neoplasms (predominantly astrocytoma) in the brain of experimental animals. In general, the incidences of tumours at the highest dose levels tested did not reach statistically significance and was within the historical control range. Therefore, no CAG level 2 was suggested for brain neoplasms.

21.2.3. CAG level 3: Mode of action

For a number of the active substances having phenomenological / specific effects on the nervous system as described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

21.2.3.1. CAG level 3a1: Modulation of the cholinergic system

Several of the active substances reported to affect the nervous system in experimental studies have shown that they are able to modulate the cholinergic transmission of nerve impulses. This mode of action is associated with the effects seen at CAG level 2a.

Acetylcholine is a neurotransmitter released from nerve endings, which allows nerve impulses to move from one nerve to another or from a nerve to the organ it controls. It is a neurotransmitter in both the peripheral nervous system (PNS) and the central nervous system (CNS) in many organisms including humans. Acetylcholine is one of many neurotransmitters in the autonomic nervous system (ANS) and the only neurotransmitter used in the motor division of the somatic nervous system (sensory neurons use glutamate and various peptides at their synapses).

In the peripheral nervous system acetylcholine acts as a transmitter between motor nerves and the fibres of skeletal muscles at all neuromuscular junctions. On release, acetylcholine acts almost instantly, to cause a sequence of chemical and physical events which cause contraction of the muscle fibre. The action of acetylcholine is terminated rapidly following its inactivation by the enzyme acetylcholineesterase (AChE).

These same principles apply to cholinergic transmission at sites other than neuromuscular junctions, although the structure of the synapses differs. In the autonomic nervous system these include nerve-to-nerve synapses at the ganglia in both the sympathetic and the parasympathetic division, and the endings of parasympathetic nerve fibres on non-voluntary organs and tissues such as (smooth) muscle, the heart, and glandular cells. In response to activation of this nerve supply, smooth muscle contracts (notably in the gut), the frequency of heart beat is slowed, and glands secrete. Acetylcholine is also an important transmitter at many sites in the brain at nerve-to-nerve synapses.

For numerous active substances, including carbamates and organophosphates, the pesticidal mode of action for killing insects is an inhibition of this enzyme. Because there is a structural similarity between insect and mammalian AChE, the insect AchE inhibiting active substances can also affect mammalian AChE.

There are two main types of cholinergic receptors, nicotinic and muscarinic acetylcholine receptors. Nicotinic acetylcholine receptors (nAChR) are located at synapses between two neurons and at synapses between neurons and skeletal muscle cells. Muscarinic acetylcholine receptors (mAChr) are located at the synapses of nerves with smooth or cardiac muscles. Each type is also located in the brain.

The active substances identified as affecting the cholinergic transmission by either inhibiting the acetylcholinesterase activity or being direct ligands of the nicotinic or muscarinic acetylcholine receptors are allocated to CAG level 3a1 ‘Modulation of the cholinergic transmission’ and are listed in Table 21.5.

Table 21.5. CAG level 3a1: Modulation of the cholinergic transmission

Acetamiprid	Fenpropidin	Phosmet
Chlormequat	Fenpropimorph	Pirimicarb
Chlorpropham	Formetanate	Pirimiphos-methyl
Chlorpyrifos	Fosthiazate	Propamocarb
Chlorpyrifos-methyl	Imidacloprid	Propineb
Deltamethrin	Indoxacarb	Prosulfocarb
Desmedipham	Mepiquat	Spinosad
Dimethoate	Methiocarb (aka mercaptodimethur)	Thiacloprid
Dimoxystrobin	Methomyl	Thiram
Ethephon (both inhibition of acetyl and buturyl-choline esterase)	Molinate	Tolclofos-methyl
Ethoprophos	Oxamyl	Ziram
Fenamiphos (aka phenamiphos)	Phenmedipham	

21.2.3.2. CAG level 3a2: Modulation of the GABA system

Gamma-aminobuturic acid (GABA) is the main inhibitory neurotransmitter in the CNS. It is formed from glutamate. GABA contributes to motor control, vision, and many other cortical functions. It also regulates anxiety. In accordance with this all substances in this group are also members of CAG level 2a (active substances that have effects on functional changes in the nervous system).

The active substance identified as affecting the GABA system is allocated to CAG level 3a2 'Modulation of the GABA system' and are listed in Table 21.6.

Table 21.6. CAG level 3a2: Modulation of the GABA system

Abamectin		

21.2.3.3. CAG level 3a3: Modulation of the dopaminergic system

Dopamine is an inhibitory neurotransmitter involved in controlling movement and posture. It also modulates mood and plays a central role in positive reinforcement and dependency. In accordance with this all substances in this group are also members of CAG level 2a (active substances that have effects on functional changes in the nervous system).

The active substances identified as affecting the dopaminergic system are allocated to CAG level 3a3 'Modulation of the dopaminergic system' and are listed in Table 21.7.

Table 21.7. CAG level 3a3: Modulation of the dopaminergic system

2,4-D	Triadimenol	Ziram
Glufosinate		

21.2.3.4. CAG level 3c1: Modulation of the glutamate system

Glutamate is a major excitatory neurotransmitter in the CNS that is associated with learning and memory. It is also thought to be associated with Alzheimer's disease, whose first symptoms include memory malfunctions. In accordance with this substances in this group should also be members of CAG level 2c (active substances that have effects on cognition). However, as already mentioned under CAG level 2c only few investigations on learning and memory performance have been performed with the active substances included in this project. Thus, the number of substances included in CAG level 3c1 is larger than described in CAG level 2c.

The active substances identified as affecting the glutamate system are allocated to CAG level 3c1 'Modulation of the glutamate system' and are listed in Table 21.8.

Table 21.8. CAG level 3c1: Modulation of the glutamate system

Glufosinate	Maneb	Ziram
Mancozeb	Thiram	

21.2.3.5. CAG level 3d1: Modulation of mitochondrial function

Several active substances (also implicated in dysregulation of the dopaminergic system) affect the function of mitochondria *in vitro*, mainly in an inhibitory fashion (Thrash et al. 2007).

The active substances identified as affecting the mitochondrial function are allocated to CAG level 3d1 and are listed in Table 21.9. It should be noted that the CAG level 3d1 is not allocated to a specific CAG level 2.

Table 21.9. CAG level 3d1: Modulation of michondrial function

2,4-D	Cypermethrin	Maneb
Chlorpropham	Mancozeb	Pyrethrins
Chlorpyrifos		

21.2.3.6. CAG level 3e1: Demyelination

Demyelination is the loss of the myelin sheath insulating the nerves and results in disruption of signals between the brain and other parts of the body.

The active substances identified as inducing demyelination are allocated to CAG level 3e1 and are listed in Table 21.10. It should be noted that the CAG level 3e1 is not allocated to a specific CAG level 2.

Table 21.10. CAG level 3e1: Demyelination

2,4-D	Fenpropidin	Maneb
Cymoxanil	Isoxaflutole	Molinate
Cypermethrin	Mancozeb	

21.2.3.7. CAG level 3f1: Neuronal degeneration

A neuron is defined as a signal conducting cell of the nervous system. Typically a neuron consists of a cell body with short radiating processes called dendrites and most neurons also possess a long projection called the axon. The axon with its sheath constitutes the nerve fiber. Neuron degeneration is the progressive loss of function or structure of this cell type.

The active substances identified as inducing neuronal degeneration are allocated to CAG level 3f1 and are listed in Table 21.11. It should be noted that the CAG level 3f1 is not allocated to a specific CAG level 2.

Table 21.11. CAG level 3f1: Neuronal degeneration

Alpha-Cypermethrin (aka alphamethrin)	Isoxaflutole	Thiacloprid
Chlorpyrifos	Maneb	Thiram
Cypermethrin	Molinate	Tri-allate
Dinocap	Oxasulfuron	Triflusulfuron
Flufenacet	Quinoclamine	Ziram

21.2.4. CAG level 4: Mechanism of action

For some of the phenomenological / specific effects on the nervous system described under CAG level 2 and the mode of actions indicated at CAG level 3, respectively, a mechanism of action has been proposed. For the remaining substances, no information regarding mechanism of action has been found and consequently, these substances cannot be allocated to a CAG level 4.

21.2.4.1. CAG level 4a1a: Acetylcholinesterase inhibition

A number of the active substances identified as affecting the cholinergic system (CAG level 3a1) were identified as being acetylcholinesterase inhibitors. These substances are allocated to CAG level 4a1a ‘Acetylcholinesterase inhibition’ and are listed in Table 21.12.

Table 21.12. CAG level 4a1a: Acetylcholinesterase inhibition

Chlorpropham	Fenpropidin	Pirimicarb
Chlorpyrifos	Fenpropimorph	Pirimiphos-methyl
Chlorpyrifos-methyl	Formetanate	Propamocarb
Desmedipham	Fosthiazate	Prosulfocarb
Dimethoate	Methiocarb (aka mercaptodimethur)	Thiram
Dimoxystrobin	Methomyl	Tolclofos-methyl
Ethephon	Molinate	Ziram
Ethoprophos	Oxamyl	
Fenamiphos (aka phenamiphos)	Phosmet	

21.2.4.2. CAG level 4a1b: Nicotinic acetylcholine receptor agonist

Some of the active substances identified as inhibiting AChE and being direct ligands of the nicotinic acetylcholine receptor (nAChR) (CAG level 3a1) were identified as being nicotinic acetylcholine receptor agonists. These substances are allocated to CAG level 4a1b 'Nicotinic acetylcholine receptor agonist' and are listed in Table 21.13.

Table 21.13. CAG level 4a1b: Nicotinic acetylcholine receptor agonist

Acetamiprid	Imidacloprid	Propineb
Chlormequat	Indoxacarb	Thiacloprid

21.2.4.3. CAG level 4a1c: Muscarinic acetylcholine receptor agonist

Two of the active substances identified as inhibiting AChE and being direct ligands of the muscarinic acetylcholine receptor (mAChR) (CAG level 3a1) were identified as being muscarinic acetylcholine receptor agonists. These substances are allocated to CAG level 4a1c 'Muscarinic acetylcholine receptor agonist' and are listed in Table 21.14.

Table 21.14. CAG level 4a1c: Muscarinic acetylcholine receptor agonist

Chlormequat	Mepiquat	

21.2.4.4. CAG level 4a2a: GABA gated chloride channel blocker

The $GABA_A$ receptor is an ionotropic receptor and ligand-gated ion channel. Its endogenous ligand is γ-aminobutyric acid (GABA), the major inhibitory neurotransmitter in the central nervous system. Upon activation, the $GABA_A$ receptor selectively conducts chloride ions (Cl^-) through its pore, resulting in hyperpolarisation of the neuron. This causes an inhibitory effect on neurotransmission. Ligands which decrease receptor activation usually have opposite effects, including anxiogenesis and convulsion.

Three of the active substances identified as affecting the GABA system (CAG level 3a2) were identified as being GABA gated chloride channel blockers. These substance are allocated to CAG level 4a2a 'GABA receptor agonist' and are listed in Table 21.15.

Table 21.15. CAG level 4a2a: GABA gated chloride channel blocker

Abamectin	Deltamethrin	Fipronil

It should be noted that abamectin has been reported to act both as a GABA receptor agonist and a GABA gated chloride channel blocker. These two mechanisma potentially act against each other with the former acting GABA like and the latter opposing the effect of GABA.

21.2.4.5. CAG level 4c1a: Glutamate NMDA receptor modulation

Glutamate acts via two classes of receptors, ligand gated ion channels (ionotropic receptors) and G-protein coupled (metabotropic) receptors. The ionotropic glutamate receptors are subdivided into three groups (AMPA, NMDA and Kainate receptors).

N-Methyl-D-aspartate (NMDA) is the specific agonist at the NMDA receptor mimicking the action of glutamate, the neurotransmitter which normally acts at that receptor. Two of the active substances identified as affecting the glutamate system (CAG level 3c1) were identified as being NMDA receptor modulators. These substances are allocated to CAG level 4c1a and are listed in Table 21.16.

Table 21.16. CAG level 4c1a: Glutamate NMDA receptor modulation

Glufosinate	Triadimenol	

21.2.4.6. CAG level 4c1b: Glutamate vesicular transport interference

Some of the active substances identified as affecting the glutamate system (CAG level 3d) were identified asfound to interfering with the vesicular transport of glutamate. These substances are allocated to CAG level 4dc2 and are listed in Table 21.17.

Table 21.17. CAG level 4c1b: Glutamate vesicular transport interference

Mancozeb	Thiram	Ziram
Maneb		

21.2.4.7. CAG level 4g1a: Voltage-gated sodium channel blocker

Voltage-gated ion channels are a class of transmembrane ion channels that are activated by changes in electrical potential difference near the channel and mediate a rapid and co-ordinated depolarisation.

Voltage-gated ion channels are especially critical in neurons, but are common in many types of cells. They have a crucial role in excitable neuronal and muscle tissues, allowing a rapid and co-ordinated depolarisation in response to triggering voltage change. Found along the axon and at the synapse, voltage-gated ion channels directionally propagate electrical signals.

Voltage-gated ion channels are composed of several subunits arranged in such a way that there is a central pore through which ions can travel down their electrochemical gradients. The channels tend to be ion-specific, although similarly sized and charged ions may sometimes travel through them.

Examples include the sodium and potassium voltage-gated channels of nerve and muscle, and the voltage-gated calcium channels that play a role in neurotransmitter release in pre-synaptic nerve endings.

Some of the active substances affecting the nervous system belonging to the pyrethroids were identified as blocking the voltage-gated sodium channel (Shafer et al. 2004). These substances are allocated to CAG level 4g1a and are listed in Table 21.18. It should be noted that the CAG level 4g1a is not allocated to a specific CAG level 2.

Table 21.18. CAG level 4g1a: Voltage-gated sodium channel blocker

Alpha-cypermethrin (aka alphamethrin)	Cypermethrin	Indoxacarb
Beta-cyfluthrin	Deltamethrin	Lambda-Cyhalothrin
Cyfluthrin	Esfenvalerate	

21.2.4.8. CAG level 4h1a: Voltage-gated delayed rectifier of potassium channels

One of the active substances affecting the nervous system was identified as a voltage-gated delayed rectifier of potassium channels. This substance is allocated to CAG level 4h1a and is listed in Table 21.19. It should be noted that the CAG level 4h1a is not allocated to a specific CAG level 2.

Table 21.19. CAG level 4h1a: Voltage-gated delayed rectifier of potassium channels

Alpha-cypermethrin (aka alphamethrin)		

21.2.4.9. CAG level 4i1a: Voltage-gated calcium channel interference

Some of the active substances affecting the nervous system belonging to the pyrethroids were identified as interfering with voltage-gated calcium channels. These substance are allocated to CAG level 4i1a and are listed in Table 21.20. It should be noted that the CAG level 4i1a is not allocated to a specific CAG level 2.

Table 21.20. CAG level 4i1a: Voltage-gated calcium channel interference

Alpha-cypermethrin (aka alphamethrin)	Cyfluthrin	Deltamethrin
Beta-cyfluthrin	Cypermethrin	

21.3. Discussion of CAGs for the nervous system

Sixty-seven active substances were identified to affect the nervous system and were allocated to CAG level 1. Three distinct CAGs at level 2 have been proposed. Information on modes/mechanisns of action is available for a number of the active substances. The information is summarised in Appendix AC.

The phenomenological / specific effects on the nervous system that are proposed at CAG level 2 may to some extent be interrelated and the individual modes of action identified at CAG level 3 may play roles in several (or sometimes all) of these effects. Although, the mechanisms of action identified at CAG level 4 constitute optimal criteria for creating CAGs, with the exception of CAG level 4a1a: Acetylcholineesterase inhibition, only a limited number of active substances have been studied for the other specific mechanisms. Therefore the CAGs at level 3 and 2 are also recommended for consideration in CRA.

21.3.1. Chemical classes as basis for CAGs for the nervous system

Active substances belonging to the same chemical class may have similar toxicological effects. Information in the DARs on effects on the nervous system is summarised below for evaluation of similarity of toxicological effects within the relevant chemical classes, i.e. the chemical classes containing more than one active substance.

21.3.1.1. Aryloxyalkanoic acids

One aryloxyalkanoic acid was identified as having a specific chronic effect on the nervous system: 2,4-D (decreased response to sharp noise and swollen axons in brain and spinal cord). After injection into basal ganglia of rats 2,4-D produced decreased locomotor activity, postural effects, increased serotonin (5-HT) and homovanillic acid (HVA) levels and diminished levels of striatal 5-HT and dopamine). The remaining aryloxyalkanoic acids (2,4-DB, dichlorprop-P, MCPA, MCPB, mecoprop, and mecoprop-P) all show similar acute clinical signs as 2,4-D, however, no specific effects on the nervous system were seen for dichlorprop-P and MCPA in the neurotoxicity studies reported in the DARs. For 2,4-DB, MCPB, mecoprop and mecoprop-P no specific studies on neurotoxicity were available. Therefore, the information in the DARs does not allow the conclusion that aryloxyalkanoic acids have similar toxic effects on the nervous system.

21.3.1.2. Benzoic acids (aromatic carboxylic acids)

Dicamba classified as a benzoic acid was identified as having an effect on the nervous system (abnormal locomotion and gait, ataxia, sporadic muscle spasms, uncoordinated movements). Benzoic acid itself, being classified as an aromatic carboxylic acid, show similar effects on the nervous system (aggressiveness, hypersensitivity, tremor, convulsions, uncoordinated movement and depression).

21.3.1.3. Benzylureas

One benzylurea was identified as having a specific effect on the nervous system: Lufenuron (episodes of spontaneous clonic-tonic convulsion or fasciculations and facilitated pentylenetetrazol-induced generalised convulsions was the only effect reported in a neurotoxicity study). For the remaining benzylureas (diflubenzuron, teflubenzuron), this effect has not been investigated. For both compounds the DARs state that special testing for neurotoxicity is not deemed necessary. Therefore, the information in the DARs does not allow the conclusion that the benzylureas have similar toxic effects on the nervous system.

21.3.1.4. Biscarbamates

Two active substance are biscarbamates. Phenmedipham was identified to increase choline esterase activity in rat brain, red blood cells and plasma. For the other biscarbamate, desmedipham, which is structurally closely related to phenmedipham, conflicting results were reported. In one study, rat brain, red blood cell and plasma choline esterase activities were reported to be reduced whereas in another (similar) rat study reduced brain activity and increased plasma activities were noted. It has to be noted that these biscarbamates are used as herbicides and although they upon metabolism have the potential to yield the structurally closely related (mono)-carbamates, they seem to increase cholinesterase activity in rats. This is in contrast to the (mono)-carbamates that are mainly insecticides that act by inhibiting the activity of cholinesterase.

21.3.1.5. Carbamates

Six carbamates (carbamate esters or urethanes) were identified as having effects in the nervous system in mammals: Chlorpropham (decreased choline esterase activity, affected surface righting, axonal degeneration, abnormal gait), methiocarb (choline esterase activity inhibition), methomyl (decreased grip strength, functional observational battery parameters, acetylcholine esterase inhibition), oxamyl (decreased forelimb and hind limb grip strength, tremor, acetylcholine esterase inhibition), pirimicarb (acetylcholine esterase inhibition), propamocarb (vacuolization of the choroid plexus, acetylcholine esterase inhibition). The remaining active substances being carbamates (benthiavalicarb, iprovalicarb) included in the CAG project had no effects on the nervous system in mammals. These two carbamates are used as fungicides in contrast to the six carbamate esters identified as having effects in the nervous system which are mainly used as insecticides and all displayed effects as acetylcholine esterase inhibitors. In addition, the carbamate moities of benthiavalicarb and iprovalicarb are very similar, but distinctly different from the carbamate moieties of the six neurotoxic carbamates. These findings suggests that being a carbamate ester does not automatically make a substance an acetylcholine esterase inhibitor.

21.3.1.6. Dithiocarbamates

Five dithiocarbamates were identified as having effects in the nervous system: Mancozeb (ataxia, abnormal gait or mobility, tremors and hindlimb paralysis, myelin damage, Schwann cells proliferation), maneb (abnormal gait and hind limb paralysis, axonal shrinkage, neurofibrillary degeneration, myelin sheath thickening, demyelination, myelin phagocytosis, myelin bubbles and Schwann cell proliferation), metiram (reduced grip strength, hind limb weakness, ataxia, increased incidence of meningo-encephalocele, hydrocephaly, obstructed neural tube), propineb (propreoceptive deficits and hind-limb wheelbarrowing), thiram (ataxia or paralysis of the hind legs, chromatolysis of motor neurones, hyperactivity, abnormal gait, demyelination, degeneration of the axis cylinders, and presence of macrophages in the nerve bundle of the sciatic nerve). Mancozeb, maneb and thiram (together with the dimethyldithiocarbamate, ziram) all interfere with vesicular transport of glutamate. Maneb and mancozeb both have effects on demyelination and neuronal degeneration. In addition mancozeb and maneb have effects on mitochondrial function and concomitantly on the dopaminergic system. Therefore, the information in the DARs allows the conclusion that the dithiocarbamates have similar toxic effects (neuropathy) on the nervous system.

21.3.1.7. Dimethyldithiocarbamates

The dimethyldithiocarbamate ziram (could also be classified as a dithiocarbamate) was identified as having an effect on the nervous system (ataxia, muscle tremors and convulsions, posture abnormalities, decreased grip strength). It is noted that ziram interfere with vesicular transport of glutamate and that this effect is analogous to the effect of the dithiocarbamates Mancozeb, maneb and thiram. In addition ziram in parallel to maneb and mancozeb have effects on the dopaminergic system. Thus, this indicates that the six dithiocarbamates could be considered together when reviewing similar nervous system effects (neuropathy) in relation to chemical groups.

21.3.1.8. Morpholines

One morpholine was identified as having an effect on the nervous system: Fenpropimorph. (reduced serum choline esterase, postural effects, decreased grip strength). For the remaining morpholines (dimethomorph, dodemorph, spiroxamine), no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that morpholines have similar toxic effects on the nervous system.

21.3.1.9. Neonicotinoids

Four neonicotinoids were identified as having effects in the nervous system: Acetamiprid (decreased food consumption, nicotinic receptor agonist), clothianidin (increased startle reflex, convulsions, and stiff movements, increased motor activity), imidacloprid (functional observational battery effects, nicotinic receptor agonist), thiacloprid (nerve degeneration, nicotinic receptor agonist). For the remaining neonicotinoid (thiamethoxam) no effects on the nervous system was reported in the DAR. In conclusion, the information in the DARs indicate that neonicotinoids to some extent have similar toxic effects on the nervous system (functional observational battery effects, nicotinic receptor agonists).

21.3.1.10. Organophosphates

Nine organophosphates were identified as having effects in the nervous system: Chlorpyrifos, chlorpyrifos-methyl, dimethoate, ethoprophos, fenamiphos (aka phenamiphos), fosthiazate, glufosinate, phosmet, pirimiphos-methyl. For the remaining active substance classified as an organophosphate (fosetyl) no effects on the nervous system was reported in the DAR. However, fosetyl is the aluminium salt of ethyl hydrogen phosphonate and is therefore an organometallic compound and not a "true" organophosphate. Eight of the nine organophosphates identified as having effects in the nervous system displayed effects as acetylcholine esterase inhibitors. The remaining organophosphate (glufosinate) interacts with the glutamate system. In contrast to the eight organophosphates with acetylcholine esterase inhibiting properties that are used as insecticides/nematicides/acaricides, glufosinate is used as a herbicide. Therefore, the information in the DARs allows the conclusion that the organophosphates that are used as insecticides/nematicides/acaricides have similar toxic effects (neuropathy) on the nervous system.

Regarding choline esterase inhibition there has been some debate as to whether to include carbamates and organophosphates in a common assessment group (UK Science Group 2004). The United States Environmental Protection Agency (US EPA) has decided to group the carbamates (N-methyl carbamates) and the organophosphates into separate common mechanism groups. This was done on the basis of differences in kinetics of the carbamylation and the phosphorylation of the acethylcholine esterase protein. In contrast to this, the UK Science Group found the use of separate groups highly questionable. The argument for having a common assessment group including both carbamates and organophosphates was that there is a potential for concurrent exposure to substances from both groups from various sources in the diet.

In agreement with the reasoning by the UK Science Group it has propsed to group the carbamates, organophosphates, morpholines, strobilurines, thiocarbamates and fenpropidin causing acetylcholinesterase inhibition into a common assessment group: CAG level 4a1a 'Acetylcholineesterase inhibition'.

21.3.1.11. Phenylpyrazoles

One phenylpyrazole was identified as having an effect on the nervous system: Fipronil. (postural abnormalities, abnormal reflex action, aggressivity or irritability, convulsions, increased motor activity). For the remaining phenylpyrazole (pyraflufen-ethyl), no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that the phenylpyrazoles have similar toxic effects on the nervous system.

21.3.1.12. Pyrethroids and pyrethrin

For eight of the nine active substances classified as pyrethroids effects on the nervous system were reported in the DARs: Alpha-cypermethrin (ataxia/abnormal gait, over-activity or hunched posture, increased sensitivity to noise), beta-cyfluthrin (temor, uncoordinated gait, axonal degeneration, motor disturbances), cyfluthrin (tremor, altered gait, brain haemorrhages, increased salivation, tremor,uncoordinated gait), cypermethrin (impaired gait, ataxia, paralysis, hypersensitivity to external stimuli, gross disorientation and convulsions,

axonal damage), deltamethrin (impaired mobility and gait, stereotypic behaviour, altered air righting reflex, altered hindlimb extensor strength, reduced grip strength, increased motor activity, choreoathetosis and effects on muscarinic and nicotinic brain receptors), esfenvalerate (hyperactivity, abnormal limb movements, tremors, seizures, ataxia, decreased grip strength, decreased ease of removal from the home cage, decreased motor activity, bizarre reactions to tail pinch and to startle response) lambda-cyhalothrin (ataxia, muscle tremors and convulsions, decreased grip strength), zeta-cypermethrin (reduction in locomotor activity, ataxia, tremors, convulsions and hypersensitivity to touch). The remaining pyrethroid (etofenprox) had no effects on the nervous system according to the DAR. However, etophenprox lacks the (vinyl)cyclopropanecarboxylate moity characteristic for the majority of the active pyrethroids. Therefore, the information in the DARs allows the conclusion that the pyrethroids that are cyclopropanecarboxylate esters have similar toxic effects on the nervous system.

The effects described in the DARs, such as motor disturbances, decreased grip strength, voltage gated sodium channel blockade and voltage sensitive calcium channel interference are in accordance with reports from the open literature. Thus, Wolansky and Harill (2008) in a review reported that all pyrethroids investigated (20 substances), regardless of structure, produced a decrease in motor activity in a variety of test protocols and six substances showed a decrease in grip strength.

It is noted that pyrethrins are classified as a biopesticide. However, as the pyrethroids are structural derivatives of the naturally occurring pyrethrins, the latter could be considered together with the pyrethroids. According to the DAR pyrethrins produces tremor and postural disturbances. Thus they have toxic effects similar to the pyrethroids on the nervous system. It is noted that the primary neurotoxic site of action for pyrethroids is the voltage dependent sodium channels in excitable membranes. In conclusion, the information in the DARs allows the conclusion that the pyrethroids and pyrethrins have similar toxic effects on the nervous system.

21.3.1.13. Quarternary ammonium compounds

Two quarternary ammonium compounds were identified as having effects on the nervous system: Chlormequat (salivation, transient inhibitory action on the neuromuscular junction) and mepiquat (tremors, impaired gait, ataxia, posture abnormalities, decreased motor activity, sedation, salivation). There are no other quarternary ammonium compounds in the CAG project. Although salivation occurs with both substances, the limited amount of data and number of quarternary ammonium compounds in the CAG project makes it difficult to make a firm conclusion on similar toxic effects of quarternary ammonium compounds.

21.3.1.14. Sulfonylureas

Two sulfonylureas were identified as having effects on the nervous system: Oxasulfuron, (axonal degeneration, findings in functional observational battery parameters) and triflusulfuron. (nerve degeneration). For the remaining sulfonylureas (amidosulfuron, azimsulfuron, bensulfuron, chlorsulfuron, ethoxysulfuron, flazasulfuron, flupyrsulfuron-methyl, imazosulfuron, iodosulfuron-methyl-sodium, metsulfuron-methyl, nicosulfuron, prosulfuron, rimsulfuron, sulfosulfuron, thifensulfuron-methyl, triflusulfuron, tribenuron,

tritosulfuron) no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that the sulfonylureas have similar toxic effects on the nervous system.

21.3.1.15. Strobilurines

One strobilurine was identified as having an effect on the nervous system: Dimoxystrobin (decreased choline esterase activity, nerve degeneration). For the remaining strobilurines (azoxystrobin, fluoxastrobin, kresoxim-methyl, pyraclostrobin, trifloxystrobin), no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that the sulfonylureas have similar toxic effects on the nervous system.

21.3.1.16. Thiocarbamates

All three thiocarbamates included were identified as having an effect on the nervous system: molinate (demyelination, nerve lesions, impaired gait, ataxia, abnormal reflex function), prosulfocarb (lower choline esterase activity, decreased activity, hunched posture), and tri-allate (impaired gait, reduced grip strength). There are no other thiocarbamates in the CAG project. Therefore, the information in the DARs indicate that the thiocarbamates have similar toxic effects on the nervous system. However, only prosulfocarb has an effect on acetyl choline esterase inhibition which would suggest a common toxic mechanism in the nervous system parallel to that seen for carbamates but the lack of this effect for molinate and tri-allate, does not substantiate a firm conclusion on a similar mode/mechanism if action of thiocarbamates on the nervous system.

21.3.1.17. Triazoles

One triazole was identified as having an effect on the nervous system: Triadimenol (dopamine-reuptake, increased motor activity, hyperactivity, decreased grip strength and foot splay). For the remaining triazoles (amitrole, difenoconazole, epoxiconazole, flusilazole, metconazole, penconazole, propiconazole, tebuconazole, tetraconazole, triticonazole), no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that the triazoles have similar toxic effects on the nervous system.

21.3.1.18. Triketones

One triketone was identified as having an effect on the nervous system: Sulcotrione (ataxia, abnormal gait and posture, reduced activity). For the remaining triketone (mesotrione), no effects on the nervous system were reported in the DAR. Therefore, the information in the DARs does not allow the conclusion that the triketones have similar toxic effects on the nervous system.

21.3.1.19. Chemical class not specified/unclassified

Two of seven unclassified substances were reported to have effect on the nervous system: Fenpropidin (decreased acetylcholine esterase activity, demyelination, hind limb paralysis),

quinoclamine (nerve degeneration). As these substances are unclassified no conclusion can be drawn on their similarity to other chemical classes in the CAG project.

21.3.1.20. Conclusion: Chemical classes as basis for CAGs for the nervous system

Based on the analysis whether active substances belonging to the same chemical class may have similar effects on the nervous system it is concluded that active substances belonging to the same chemical class in general do not have similar effects on the nervous system.

However, the active substances in the following chemical classes have similar toxic effects on the nervous system: Dithiocarbamates, organophosphates, and pyrethroids and pyrethrins. Therefore, CAG for each of these three chemical classes could be proposed.

21.4. Recommended CAGs for the nervous system

The following CAGs are recommended for CRA for effects on the nervous system:

- CAG level 4a1a: Acetylcholinesterase inhibition, see Table 21.12.
- CAG level 4a1b: Nicotinic receptor agonist, see Table 21.14.
- CAG level 4a1c: Muscarinic receptor agonist, see Table 21.19.
- CAG level 4a2a: GABA gated chloride channel blocker, see Table 21.15.
- CAG level 4c1a: Glutamate NMDA receptor modulation, see Table 21.16.
- CAG level 4c1b: Glutamate vesicular transport interference, see Table 21.17.
- CAG level 4g1a: Voltage gated sodium channel blocker, see Table 21.18.
- CAG level 4i1a: Voltage gated calcium channel interference, see Table 21.20.
- CAG level 3a1: Modulation of the cholinergic system, see Table 21.5.
- CAG level 3a3: Modulation of the dopaminergic system, see Table 21.7.
- CAG level 3c1: Modulation of the glutamate system, see Table 21.8.
- CAG level 3d1: Mitochondrial function, see Table 21.9.
- CAG level 3e1: Demyelination, see Table 21.10.
- CAG level 3f1: Neuronal degeneration, see Table 21.11.
- CAG level 2a: Functional changes related to the motor division, see Table 21.2.
- CAG level 2f: Effects of reflex action, see Table 21.3.
- CAG level 2c: Effects on cognition, see Table 21.4.

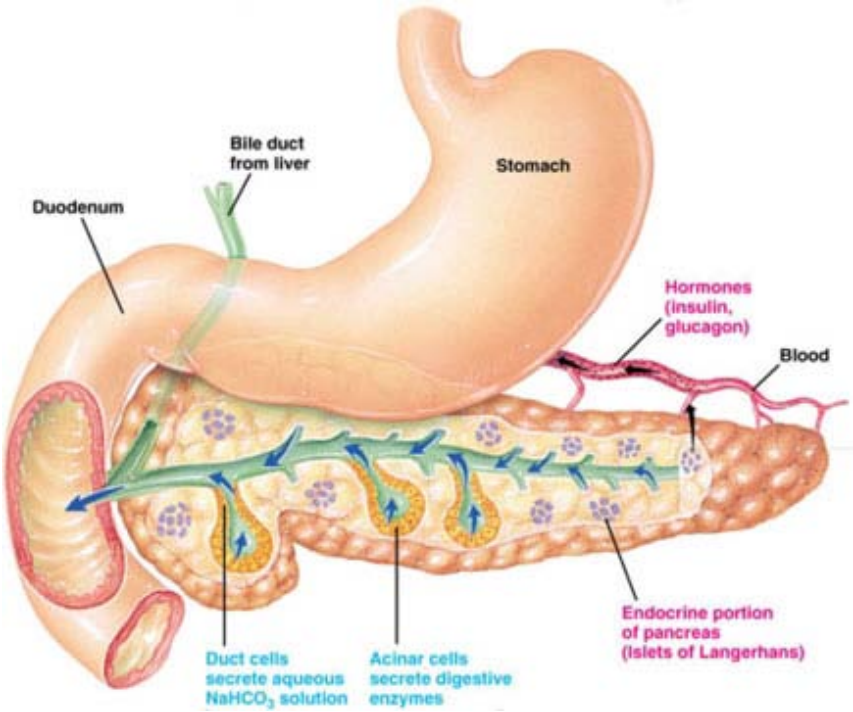
The following CAG is not recommended for CRA for the time being. However, this CAG may become relevant in the future provided that new information on other active substances justifies the mode of action / phenomenological effects forming the basis for this CAG:

- CAG level 4h1a: Voltage gated delayed rectifier of potassium channels, see Table 21.19.
- CAG level 3a2: Modulation of the GABA system, see Table 21.12.

22. Pancreas

22.1. Introduction

The pancreas serves as an endocrine gland that produces hormones and as an exocrine gland that produces digestive enzymes. The pancreas is located behind the stomach between the spleen and the duodenum.



From http://www.gopetsamerica.com/anatomy/pancreas.aspx

Figure 22.1. Anatomy of the pancreas

As can be seen in Figure 22.1 the **exocrine pancreas** is composed of acinar cells that secrete digestive enzymes and a network of ducts that secrete alkaline fluids. The digestive enzymes pass to the small intestine where they help to break down carbohydrates, proteins, and fats in the chyme. The major proteases are trypsinogen and chymotrypsinogen. Secreted to a lesser

degree are pancreatic lipase and pancreatic amylase. Trypsinogen is an inactivated form of trypsin, and chymotrypsinogen is an inactivated form of chymotrypsin. The pancreas synthesizes and stores these inactive enzymes in zymogen granules to avoid autodegradation. Once released in the intestine, an intestinal enzyme activates the inactive proteases. The exocrine pancreas also secretes alkaline fluids in order to neutralize the acidic chyme that the stomach releases into the duodenum.

Control of the exocrine function of the pancreas is both via hormones and the nervous system. Cholecystokinin is the primary hormone signaling for secretion of digestive enzymes and secretin is the primary hormone signaling for secretion of alkaline fluids. The parasympathetic nerveous system generally stimulates pancreatic secretion whereas the sympathetic nerveous system inhibits it.

Regions of **endocrine cells** are found within the exocrine pancreas. These regions are termed the islets of Langerhans named after the pathologists that discovered them. The islets of Langerhans constitute only a few percent of the mass of the pancreas. Hormones produced in the islets of Langerhans are secreted directly into the blood flow by at least five different types of cells. The hormones of which insulin is the most well know help to regulate the carbohydrate, fat and protein metabolism within the body. The secretion of insulin is regulated by chemical, hormonal and neural control. One of the effects of insulin is to facilitate uptake of glucose into the cells.

22.2. Establishment of CAGs for toxicity to the pancreas

Several active substances were identified to affect the pancreas. The effects reported include:

- Increased (relative) weight
- Hypertrophy / hyperplasia
- Atrophy / cell degeneration / necrosis
- Inflammation

The predominant effects reported in the DARs are increased weight and hypertrophy. Generally, the overall study NOAELs and LOAELs are lower than for the specific ‘pancreatic NOAEL’ and ‘pancreatic LOAEL’. Moreover, effects on the pancreas were generally observed only in one study for a particular active substance – often a long-term study and therefore, the findings were often considered in the DARs to be age-related – not treatment-related. Thus, the pancreas seems not to be a primary target organ for the active substances included in this project.

Overall, CRA for effects on the pancreas are not considered relevant. Therefore, the pancreas is not considered further for CAGs in this project.

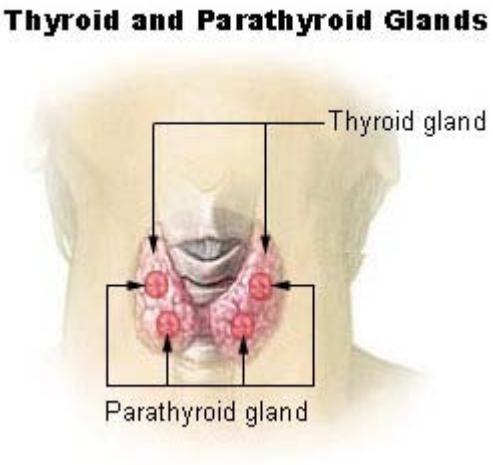
22.3. Recommended CAGs for the pancreas

No CAGs for toxicity to the pancreas is recommended.

23. Parathyroid glands

23.1. Introduction

The parathyroid glands are small endocrine glands that produce parathyroid hormone. Humans usually have four parathyroid glands, which are usually located on the rear surface of the thyroid gland – see Figure 23.1.



From: http://en.wikipedia.org/wiki/Parathyroid_gland

Figure 23.1. The thyroid and parathyroid glands

Parathyroid hormone takes part in controlling the amount of calcium in the blood and within the bones. As can be seen from Figure 9.1 (in chapter 9) the metabolism of calcium and phosphate is regulated by a complex interplay mainly between parathyroid hormone, vitamin D and calcitonine. Acting together, these substances determine the amount of dietary calcium and phosphate absorbed from the intestine, the reabsorption and excretion of calcium and phosphate by the kidney, and the deposition and release of calcium and phosphate from the bone. Both parathyroid hormone and active vitamin D stimulate osteoclasts to resorb bone (and release calcium and phosphate from bone) whereas calcitonin suppresses calcium release from bone by suppression of osteoclasts.

The production of parathyroid hormone, active vitamin D and calcitonin is mainly regulated by the serum level of calcium but also by other minerals and other substances.

23.2. Establishment of CAGs for toxicity to the parathyroid glands

23.2.1. CAG level 1: Toxicity to parathyroid glands

The active substances identified as having an effect on the parathyroid glands in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 23.1.

Table 23.1. CAG level 1: Toxicity to the parathyroid glands

Cinidon ethyl	Mepanipyrim	Sulfosulfuron
Desmedipham	Metconazole	Thiophanate-methyl
Dimethenamid-P	Picloram	
Flazasulfuron	Quinoclamine	

23.2.2. CAG level 2: Phenomenological / specific effects on the parathyroid glands

Two types of effects on the parathyroid glands were identified as a basis for establishing CAGs at level 2. Based on these effects, two distinct CAGs at level 2 are proposed. More information is given in Appendix AD.

23.2.2.1. CAG level 2a: Hypertrophy / hyperplasia

Hypertrophy is an increased size of cells and hyperplasia is an increased number of cells. Hypertrophy and hyperplasia frequently occur together and for the purpose of the CAG project these terms are allocated to a single CAG level 2, termed ‘CAG level 2a: Hypertrophy / hyperplasia’.

The active substances identified as inducing hyperplasia (hypertrophy was not reported) are allocated to CAG level 2a ‘Hypertrophy / hyperplasia’ and are listed in Table 23.2.

Table 23.3. CAG level 2a: Hypertrophy / hyperplasia in the parathyroid glands

Cinidon ethyl	Mepanipyrim	Sulfosulfuron
Desmedipham	Metconazole	Thiophanate-methyl
Dimethenamid-P	Picloram	
Flazasulfuron	Quinoclamine	

23.2.2.2. CAG level 2b: Neoplasms

One active substance was identified as inducing tumours in the parathyroid glands. This substance is allocated to CAG level 2b and is listed in Table 23.3.

Table 23.3. CAG level 2b: Neoplasms in the parathyroid glands

Cinidon ethyl		

23.2.3. CAG level 3: Mode of action

23.2.3.1. CAG level 3: Chronic nephropathy

In early chronic renal failure, excreted phosphate levels decrease and the plasma phosphate concentration increases. Plasma phosphate binds calcium resulting in hypocalcaemia. As a consequence, the parathyroids start to secrete parathyroid hormone to try to get the calcium and phosphate levels back to normal. The increased activity of the parathyroid gland may lead to hyperplasia and tumours.

For eight of the active substances identified as inducing hyperplasia in the parathyroid gland and allocated to CAG level 2a, as well as for the active substance inducing tumours in the parathyroid glands and allocated to CAG level 2b, the mode of action is considered to be secondary to chronic nephropathy and is thus an indirect effect. These substances are allocated to CAG level 3 and is listed in Table 23.4.

Table 23.4. CAG level 3: Hyperplasia / neoplasms secondary to chronic nephropathy

Cinidon ethyl	Metconazole	Sulfosulfuron
Flazasulfuron	Picloram	Thiophanate-methyl
Mepanipyrim	Quinoclamine	

For the two remaining substances, desmedipham and dimethenamid-P, no mode of action was identified to form the basis for a CAG level 3 for toxicity to the parathyroid gland.

23.2.4. CAG level 4: Mechanism of action

No information on mechanism(s) of action have been found for any of the active substances identified as having an effect on the parathyroid glands.

23.3. Discussion of CAGs for the parathyroid glands

Ten active substances were identified to have effects on the parathyroid glands and were allocated to CAG level 1. Two distinct CAGs at level 2 have been proposed. Information on mode of action is available for most of the active substances. No information on the mechanism(s) of action is available for any of them. The information is summarised in Appendix AE.

For eight of the ten active substances allocated to CAG level 2a, as well as for the active substance allocated to CAG level 2b, the available information on mode of action indicates that the hyperplasia / neoplasms is an indirect effect in the parathyroid glands, i.e., are secondary to chronic nephropathy (CAG level 3). The CAG level 3 as well as the CAG level 2a for these substances are therefore, not considered relevant in terms of CRA for a direct effect on the parathyroid glands.

As no information regarding the mode / mechanism(s) of action for the remaining two active substance allocated to CAG level 2a has been found, the CAG level 2a could be considered for CRA for effects on the parathyroid glands for these two substances.

23.4. Recommended CAGs for the parathyroid glands

The following CAG at level 2 is recommended for CRA for effects on the parathyroid glands (only for desmedipham and dimethenamid-P for the time being):

- CAG level 2a: Hypertrophy / hyperplasia, see Table 23.2.

24. Pituitary gland

24.1. Introduction

The pituitary gland (or hypophysis) is an endocrine gland located at the base of the brain and is attached to the hypothalamus (a part of the brain that affects the pituitary gland) by nerve fibers. While the pituitary gland sometimes is called the ‘master’ gland of the endocrine system, because it controls the functions of the other endocrine glands, the pituitary gland is under the control of the hypothalamus. The pituitary gland consists of three parts:

- The anterior lobe
- The intermediate lobe
- The posterior lobe

The pituitary gland secretes nine endocrine hormones that regulate homeostasis.

The anterior lobe (or adenohypophysis) secretes the following hormones:

- Growth hormone (or somatotropin) – to stimulate growth
- Prolactin – to stimulate milk production after giving birth
- ACTH (adrenocorticotropic hormone) – to stimulate the adrenal glands
- TSH (thyroid-stimulating hormone) – to stimulate the thyroid gland
- FSH (follicle-stimulating hormone) – to stimulate the ovaries and testes
- LH (luteinizing hormone) – to stimulate the ovaries or testes

The intermediate lobe secretes the following hormone:

- Melanocyte-stimulating hormone – to control skin pigmentation

The posterior lobe (or neurohypophysis) secretes the following hormones:

- ADH (antidiuretic hormone) – to increase absorption of water into the blood by the kidneys
- Oxytocin – to contract the uterus during childbirth and stimulate milk production

24.2. Establishment of CAGs for toxicity to the pituitary gland

Many active substances were identified to affect the pituitary gland. The effects reported include:

- Increased (relative) weight
- Hypertrophy / hyperplasia
- Decreased (relative) weight
- Atrophy / cell degeneration
- Cysts
- Neoplasms

The predominant effects reported in the DARs are increased weight, hypertrophy, cysts and neoplasms. Generally, the overall study NOAELs and LOAELs are lower than for the specific 'pituitary gland NOAEL' and 'pituitary gland LOAEL'. Moreover, effects on the pituitary gland were generally observed only in one study for a particular active substance – often a long-term study and therefore, the findings were often considered in the DARs to be age-related – not treatment-related. Thus, the pituitary gland seems not to be primary target organ for the active substances included in this project.

Overall, CRA for effects on the pituitary gland are not considered relevant. Therefore, the pituitary gland is not considered further for CAGs in this project.

24.3. Recommended CAGs for the pituitary gland

No CAGs for toxicity to the pituitary gland is recommended.

25. Reproductive and developmental toxicity

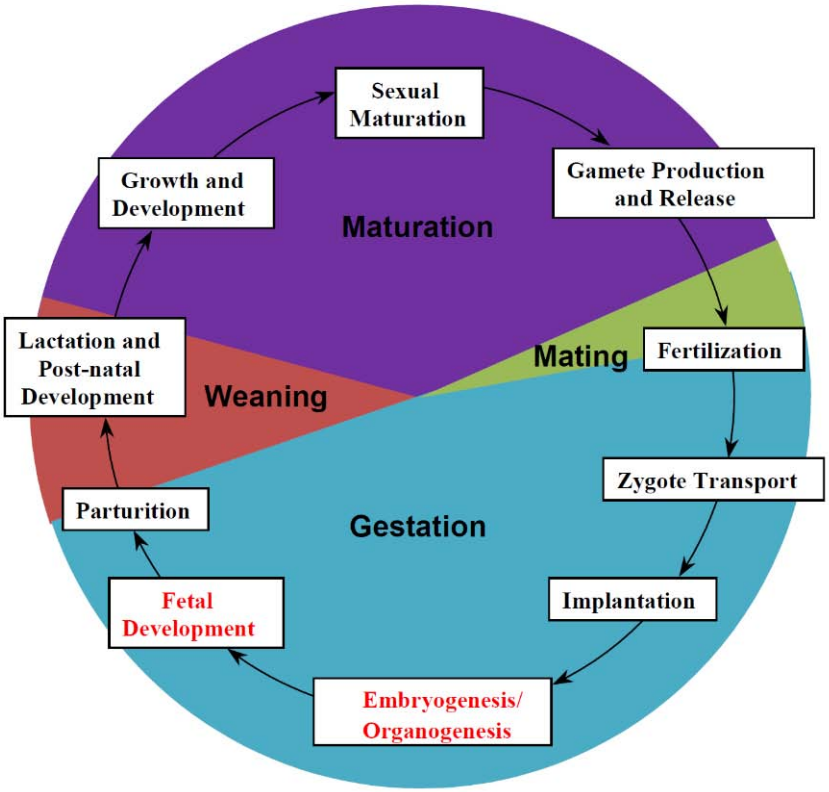
25.1. Introduction

Reproductive toxicity at large includes both male and female fertility effects and developmental toxicity effects. In some cases it is not possible to decide whether an observed effect is a sign of impaired fertility or developmental toxicity (or both). Therefore, it is decided to describe reproductive and developmental effects together in this section. As tumours in reproductive organs may be caused by similar modes of action as other effects in reproductive organs (endocrine related effects), tumours are also included in this section.

This section presents first an overview of studies on reproductive and developmental endpoints, and then an introduction to methods applied in the determination of CAGs followed by a presentation of CAGs at level 2. CAGs at level 3 and level 4 are presented subsequently together with an overview of modes and mechanisms of action leading to effects on reproduction and development. Finally, a discussion and some concluding remarks on CAGs for reproductive and developmental effects are presented.

Developmental toxicity is investigated in prenatal developmental (teratology) studies and in one- or multi-generation studies.

Reproductive toxicity i.e. effects on reproductive organs and fertility is investigated in one- or multi-generation studies. Such studies may also reveal developmental toxicity of a test substance. In addition, some of the repeated dose studies also include end points relevant for reproductive effects.



Modified from Figure 20-1 in Foster et al., 2008

Figure 25.1. The reproductive cycle. Modified from Foster et al., 2008.

Figure 25.1 illustrates how reproductive studies in various periods of life can be applied to examine effects on different endpoints. Chemical exposure during mating or early gestation may reveal effects on fertilization, implantation and early embryogenesis, whereas chemical exposure in late gestation reveals effects on foetal development. Effects of gestational exposure may be apparent not only in foetal life, but also in the postnatal period and adulthood. Chemical exposure during weaning and/or maturation may also influence adult reproductive function. In repeated dose studies chemical exposure can start before or after sexual maturation, and may reveal effects on reproductive organs and -function. It is important to keep in mind that the reproductive cycle starts at gamete production in the parents, when they were foetuses and ends after sexual maturation.

Various guideline studies are used to test effects on development and reproduction. The purpose of prenatal developmental toxicological studies (e.g. OECD 414) is to evaluate compounds for their effect on the developing foetus. The rat and rabbit are the preferred species for these studies. These tests may be integrated into the multigenerational tests (e.g. OECD TG 416) during the evaluation of reproductive toxicity. Manifestations of toxicity to the foetus are categorized as death, structural anomaly, altered or retarded growth, or functional deficiencies. At necropsy, reproductive tracts and foetuses are collected for counts of live/dead foetus and for gross and histological examination of the foetus and reproductive organs (Hamm et al., 2006).

The general purpose of generation studies is to examine successive generations to identify possible increased sensitivity to a chemical, effects on the fertility of male and female animals, pre-, peri- and postnatal effects on the mother. There are some limitations in generation studies concerning end points such as neonatal death and malformations which may not be detected, since the rat may eat dead or serious malformed pups subsequent to birth. The effect may therefore only be indirectly indicated by a decrease in litter size (Hass, 1992). In a prenatal developmental toxicity study the demonstration that intra-uterine death after implantation has occurred (resorptions) could be detected. Two-generation reproduction studies are aimed to provide general information concerning the effects of a test substance on the integrity and performance of the male and female reproductive systems, including gonadal function, the oestrus cycle, mating behaviour, conception, gestation, parturition, lactation, and weaning, and the growth and development of the offspring (OECD TG 416). The study may also provide information about the effects of the test substance on neonatal morbidity, mortality, and preliminary data on prenatal and postnatal developmental toxicity and serve as a guide for subsequent tests. In addition to studying growth and development of the F1 generation, studies are intended to assess the integrity and performance of the male and female reproductive systems as well as growth and development of the F2 generation. Some of the DARs contain old two generation studies (before update in 2001) meaning that e.g. assessment of oestrus cycle and semen quality is not included.

Repeated dose studies may also contribute with information on toxicological effects on the reproductive system, as they are intended to investigate effects on a very broad variety of potential targets of toxicity. Short-term (28-day) studies provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time, including effects on the nervous, immune and endocrine systems (OECD TG 407, Repeated Dose 28-Day Oral Toxicity Study in Rodents, updated in 2008). Sub-chronic (90-day) studies provide information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into adulthood (OECD TG 408, updated 1998). Chronic toxicity (1 or 2 years) studies are aimed at providing information on chronic toxicological effects and tumour development including tumours in the reproductive system.

25.2. Establishment of CAGs for reproductive and developmental toxicity

25.2.1. CAG level 1: Reproductive and developmental toxicity

Reproductive toxicity at large includes both male and female fertility effects and developmental toxicity effects. In some cases it is not possible to decide whether an observed effect is a sign of impaired fertility or developmental toxicity (or both). One example is decreased litter size observed in two-generation studies as this may be due to e.g. decreased fertilization (fertility effect) or foetal death (developmental toxicity). Another example is peri-natal mortality, which may be due to impaired parturition in the dams (fertility effect) or effects on the foetus/new born offspring (developmental toxicity). Consequently, it was decided to evaluate reproductive toxicity overall and not divide this into separate columns with fertility and developmental toxicity. This results in one "level 1" category for reproductive toxicity including both effects on fertility and developmental toxicity.

A total of 196 active substances have shown reproductive and/or developmental toxicity and/or induction of tumours in reproductive organs in experimental animals and thus could form a CAG level 1. However, as this CAG comprises several different types of specific effects with varying modes of action, the usefulness of this CAG is limited to very general first-steps in cumulative risk assessment.

Table 25.1. CAG level 1: Reproductive and developmental toxicity and tumours in reproductive organs

1-Methyl-cyclopropene	Deltamethrin	Glyphosate (incl trimesium	Pymetrozine
2,4-D	Desmedipham	aka sulfosate)	Pyraclostrobin
2,4-DB	Dicamba	Imazalil (aka enilconazole)	Pyrimethanil
2-Phenylphenol (incl.	Dichlorprop-P	Imazaquin	Pyriproxyfen
sodium salt orthophenyl	Difenoconazole	Imazosulfuron	Quinoclamine
phenol)	Diflubenzuron	Imidacloprid	Quinoxyfen
Abamectin (aka avermectin)	Diflufenican	Indoxacarb#	Quizalofop-P-ethyl
Acetamiprid	Dimethachlor	Iodosulfuron-methyl-sodium	Quizalofop-P-tefuryl
Acibenzolar-S-methyl	Dimethenamid-P	Ioxynil	Rimsulfuron (aka renriduron)
(benzothiadiazole)	Dimethoate	Iprodione	S-Metolachlor
Aclonifen	Dimethomorph	Iprovalicarb	Silthiofam
Amidosulfuron	Dimoxystrobin	Isoproturon	Sodium 5-nitroguaiacolate

Amitrole (aminotriazole)	Dinocap	Isoxaflutole	Sodium hypochlorite
Azimsulfuron	Diuron	Lenacil	Sodium o-nitrophenolate
Azoxystrobin	Dodemorph	Linuron	Sodium p-nitrophenolate
Beflubutamid	Epoxiconazole	Lufenuron	Spinosad
Benalaxyl	Esfenvalerate	MCPA	Spiroxamine
Benfluralin	Ethephon	MCPB	Sulcotrione
Bensulfuron	Ethofumesate	Mancozeb	Tebuconazole
Bentazone	Ethoprophos	Maneb	Tebufenpyrad
Benthiavalicarb	Ethoxysulfuron	Mecoprop	Tepraloxydim
Benzoic acid	Etofenprox	Mecoprop-P	Tetraconazole
Beta-Cyfluthrin	Etoxazole	Mepanipyrim	Thiabendazole
Bifenox	Famoxadone	Mepiquat	Thiacloprid
Boscalid	Fenamidone	Mesotrione	Thiamethoxam
Bromoxynil	Fenamiphos (aka	Metamitron	Thifensulfuron-methyl
Calcium phosphide	phenamiphos)	Metconazole	Thiophanate-methyl
Captan	Fenhexamid	Methiocarb (aka	Thiram
Carbendazim	Fenoxaprop-P	mercaptodimethur)	Tolclofos-methyl
Carfentrazone-ethyl	Fenpropidin	Metiram	Tolylfluanid
Chloridazon (aka pyrazone)	Fenpropimorph	Metrafenone	Tralkoxydim
Chlormequat (chloride)	Fenpyroximate	Metribuzin	Tri-allate
Chlorothalonil	Fipronil	Metsulfuron-methyl	Triadimenol
Chlorotoluron	Flazasulfuron	Milbemectin	Triasulfuron
Chlorpropham	Fluazinam	Molinate	Tribenuron (aka
Chlorpyrifos	Fludioxonil	Oxadiazon	metometuron)
Chlorpyrifos-methyl	Flufenacet (formerly	Oxamyl	Triclopyr
Chlorsulfuron	fluthiamide)	Oxasulfuron	Trifloxystrobin
Cinidon ethyl	Flumioxazin	Penconazole	Triflusulfuron
Clodinafop	Fluopicolide	Phenmedipham	Trinexapac (aka cimetacarb
Clofentezine	Fluoxastrobin	Phosmet	ethyl)
Clomazone	Flupyrsulfuron-methyl	Picolinafen	Triticonazole
Clopyralid	(DPX KE 459)	Pirimicarb	Tritosulfuron
Clothianidin	Fluroxypyr	Pirimiphos-methyl	Ziram
Copper compounds	Flusilazole	Propamocarb	Zoxamide
Cyazofamid	Folpet	Propaquizafop	lambda-Cyhalothrin
Cyclanilide	Forchlorfenuron	Propiconazole	zeta-Cypermethrin
Cyflufenamid	Formetanate	Propineb	
Cyfluthrin	Fosetyl	Propoxycarbazone	
Cymoxanil	Fosthiazate	Propyzamide	
Cypermethrin	Fuberidazole	Prosulfocarb	
Cyprodinil	Gibberellin	Prosulfuron	
Cyromazine	Glufosinate	Prothioconazole	

25.2.2. CAG level 2: Phenomenological / specific effects for reproductive and developmental toxicity

The above mentioned CAG level 1 active substances were allocated to various CAGs at level 2 based on their 'specific effects'. In order to facilitate this sorting of the substances, the following terminology for specific effect groups was developed:

- *Body weight* + decreased/increased + foetus/pups + age (e.g. birth weight, from PND 14 etc.). Only effects in offspring were included

- *Delayed development* + type + foetus/pups + male/female if relevant (e.g. delayed ossification, foetus GD 21; delayed sexual maturation, pups, female etc.)
- *Malformations* + type of malformation + foetus/pups + age (e.g. hydrocephalus, foetus GD 21; hypospadias, adult male offspring etc.)
- *Variations* + type + foetus/pups (e.g. extra ribs, GD 21 foetus etc.)
- *Death* + foetus/pups + type/age (e.g. post implantation loss; pups, during lactation etc.)
- *Offspring*, other effects + type (e.g. histopathology; functional effects etc.)
- *Fertility* + type + male/female (e.g. fertility index; time to pregnancy; semen quality; effect on weight or histopathology of reproductive organs; impaired parturition etc.). Only effects in adult animals, e.g. parental generation (F0) or in repeated dose toxicity studies, were included
- *Tumour* + site + type (e.g. tumour, testes, Leydig cell)

Each of these specific effect groups were further subdivided into CAGs at level 2 as outlined in the following.

For each study, species, strain and mode of administration was noted together with a note on either study duration (for repeated dose studies, e.g. 28 days, 90 days, 2 years) or type of study (two-generation study, one-generation study, prenatal developmental toxicity study). For non-guideline studies the dosing period was noted (e.g. GD 13-18, GD 7-PND 16). This information can be found in the Access database, but is not included for each category.

All effects listed are in vivo effects from studies accepted by the DAR and related documents (ECCO, EFSA peer review) and mainly statistically significant effects are noted. However, in some cases the DAR did not contain sufficient information on statistical analyses, and effects considered relevant by the DAR are listed anyway. Also effects that were considered relevant by the DAR but were not statistically significant were included.

For some studies the describtion in DARs did not note NOAELs/LOAELs in mg/kg bw/day but as ppm in diet. In those cases NOAELs/LOAELs were converted as described in the above general section on collection of information, and the remark “converted from ppm” was included in the Remarks column of the Access database.

For developmental studies it may be relevant to consider whether effects on offspring are seen above, below or at maternal effect levels, i.e. whether developmental NOAELs are above, below or at maternal NOAELs. The letter “t” is used in the CAG tables in Appendices AP to AX as an indication of cases where the relevant developmental NOAEL is at or above the maternal NOAEL most often for decreased body weight and/or decreased food consumption.

The letter “m” is presented in the CAG table in Appendices AP to AX for studies performed on metabolites. In some cases, both mother compound and metabolite have the same effect, and therefore two lines are presented for one active substance in the CAG table.

Studies from the open literature are included when these appeared to be of a sufficient quality and relevance. Mainly Pubmed was used for literature searches as this is the most comprehensive database covering topics relevant for human health. When no or little

information was found for a certain substance, further searches were performed in DTU digital library, a search machine collecting abstracts from Biosis, Food Science and Technology Abstracts, Web of Science, Pubmed, Inspec, Compendex, Academic Search Elite/Ebsco, and Business Source Premier/Ebsco.

The search strategy for studies on reproduction and development in open literature was as follows:

1. Search compound name

a. If few hits (less than 100): check for studies on reproductive and developmental toxicity.

b. If many hits (more than 100): go to 2

2. Search compound name and one of the following words: rats, mice, *in vitro*, endocrine, reproduction, fertility, development

a. Check for studies on reproductive and developmental toxicity or mechanisms of action relevant to reproduction and development

This approach resulted in a number of "level 2" categories that can be used for selection of CAGs. Six major groups were identified, some of them containing more than one major CAG at level 2:

- 2a: Delayed development and decreased (increased) body weight
- 2b: Malformations and variations
- 2c: Pre- and postnatal death
- 2d: Other effects in offspring
- 2e: Fertility
- 2f: Tumours in reproductive organs

An observed effect can be included in more than one "level 2" category (e.g. decreased litter size can be found in both the "death" and the "fertility" or the "offspring, other effects" categories). Using the above terminology, effect categories could be made and grouped in "tree structures" representing various levels of detail. For some level 2 effect categories it was possible to make level 3 subgroups based on knowledge of chemical mode of action. Examples are delayed development and low body weight in offspring caused by thyroid disruption, or male reproductive effects caused by anti-androgenicity. For some anti-androgenic chemicals, organ weight changes and offspring effects the mechanism of action could be attributed to androgen receptor antagonism (based on data from open literature), and this would be an example of a level 4 subgroup.

The following figure (Figure 25.2) presents CAG level 2 categories for specific effects within reproductive and developmental toxicity. Some of the level 2 categories are grouped, as these categories are likely to be interrelated.

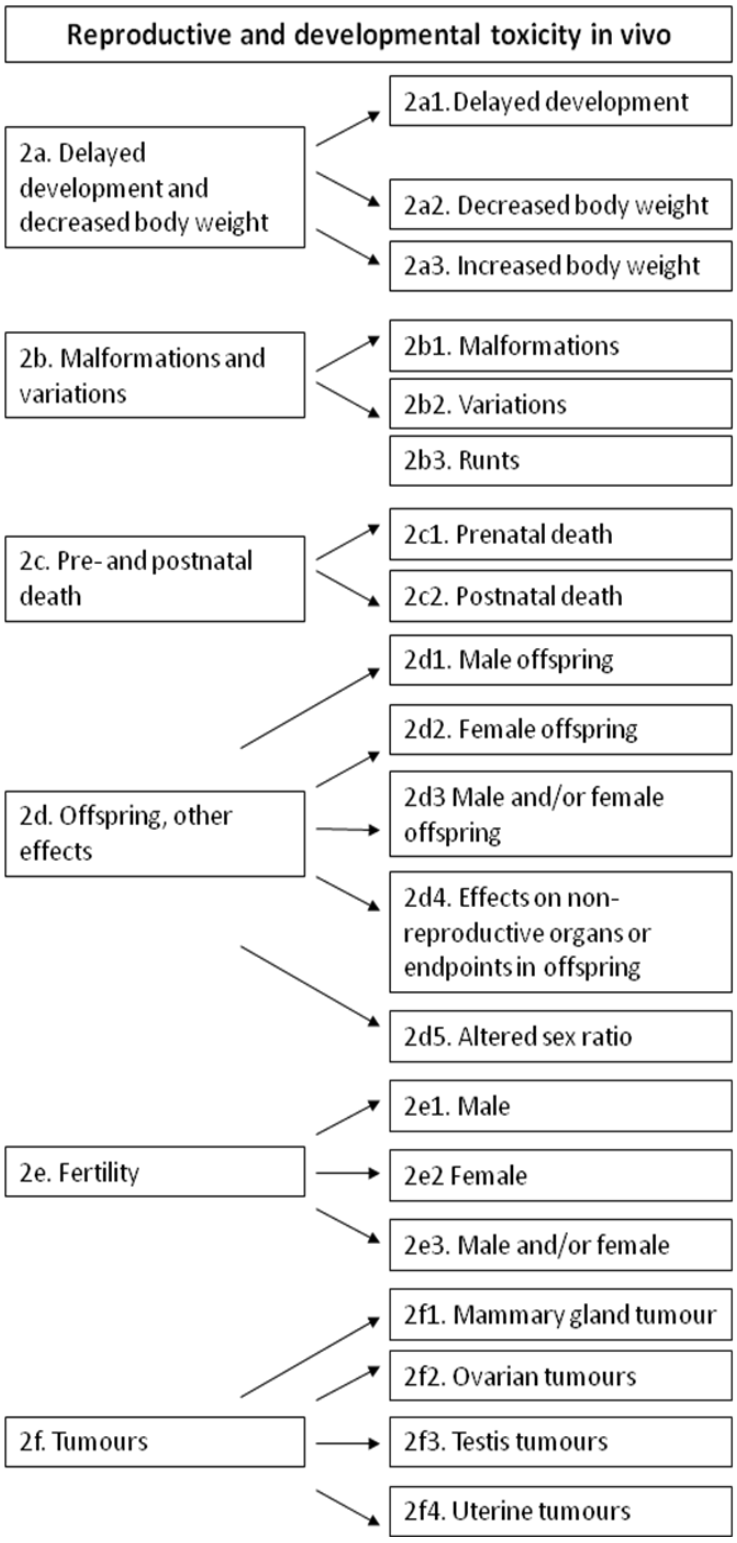


Figure 25.2. Main CAGs for reproductive and developmental toxicity.

These main CAGs are further subdivided. In the following text CAGs are numbered systematically with the first letter indicating main the level 2 CAG (e.g. 2a "Delayed development and decreased body weight"); the following number indicating main subgroup (e.g. 2a1 "Delayed development"); the following letter indicating further subgrouping (e.g. 2a1a "Delayed prenatal development" and 2a1b "Delayed postnatal development"), and in some cases yet another number indicates additional subgrouping (e.g. 2a1b1 "Delayed sexual maturation" and 2a1b2 "Delayed eye opening").

Each section on main CAGs contains tables describing these subdivisions listing which specific effects are included in each CAG. However, the following list shows all level 2 subgroups for use as an overview and reference for the section on Reproductive and developmental toxicity as a whole. CAG names in *italics* are searchable in the Access database (in column "phenomenological/specific effect CAG level 2") using specifically the listed words.

2a. Delayed development and decreased body weight

1. Delayed development
    - a. *Delayed prenatal development*
        - *1. Delayed ossification*
        - *2. Delayed tooth eruption*
        - *3. Delayed development (other)*
    - b. *Delayed postnatal development*
        - *1. Delayed sexual maturation*
        - *2. Delayed eye opening*
        - *3. Delayed auditory canal opening*
        - *4. Delayed pinna unfolding*
        - *5. Delayed tooth eruption*
        - *6. Delayed development (other)*
2. Decreased body weight
    - a. *Prenatal body weight decrease*
    - b. *Postnatal body weight decrease*
    - c. *Decreased body weight of adult offspring*
3. Increased body weight
    - a. *Prenatal body weight increase*
    - b. *Postnatal body weight increase*
    - c. *Increased body weight of adult offspring*

2b. Malformations and variations

1. *Malformations*
    - a. *Skeletal malformations*
    - b. *Cleft palate*
    - c. *Hydrocephalus*
    - d. *Excencephaly*
    - e. *Kidney malformations*

f. Eye malformations
g. Heart malformations

2 Variations
a. Skeletal variations
b. Urinary tract variations
c. Heart variations
d. Eye variations
e. Dilated brain vesicles

3 Runts

2c. Pre- and postnatal death

1. Prenatal death
2. Postnatal death

2d. Other effects in offspring

1. Male offspring
   a. Changes in reproductive organs of male offspring
      1. Decreased weight of male reproductive organs of offspring
      2. Reduced semen quality of offspring
      3. Change in anogenital distance of male offspring
      4. Nipple retention in male offspring
   b. Impaired fertility of male offspring
2. Female offspring
   a. Changes in reproductive organs of female offspring
   b. Impaired fertility of female offspring
3. Male and/or female offspring
   a. Impaired fertility of male and/or female offspring
4. Effects on non-reproductive organs or endpoints in offspring
   a. Changes in other organs of offspring
   b. Changes in non-reproductive endpoints in offspring
5. Altered sex ratio

2e. Fertility

1. Male
   a. *Changes in male reproductive organs*
      1. Decreased weight of male reproductive organs and *Small male reproductive organs*
      2. Decreased weight of male reproductive organs in Hershberger assay
      3. Reduced semen quality
      4. Leydig cell hyperplasia
   b. Impaired male fertility
2. Female
   a. Changes in female reproductive organs
   b. Impaired female fertility
3. Male and/or female

a. Impaired male and/or female fertility

2f. Tumours in reproductive organs

1. *Mamma tumours*
2. *Ovary tumours*
3. *Testis tumours*
4. *Uterus tumours*

25.2.2.1. CAG level 2a: Delayed development and decreased body weight

These two main CAGs are interrelated, as decreases in offspring body weights often may cause delay in pre- or postnatal development. Furthermore, decreases in offspring body weights and delayed development may have the same underlying causes. Increased offspring body weights have been found after exposure to a few active substances, which forms its own CAG.

Table 25.2. CAG level 2a: Delayed development and decreased body weight (194 compounds)

2,4-D	Ethofumesate	Oxadiazon
2,4-DB	Ethoprophos	Oxamyl
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Ethoxysulfuron	Oxasulfuron
Abamectin (aka avermectin)	Etofenprox	Penconazole
Acetamiprid	Etoxazole	Phenmedipham
Acibenzolar-S-methyl (benzothiadiazole)	Famoxadone	Phosmet
Aclonifen	Fenamidone	Picolinafen
Amidosulfuron	Fenamiphos (aka phenamiphos)	Pirimicarb
Amitrole (aminotriazole)	Fenhexamid	Pirimiphos-methyl
Azimsulfuron	Fenoxaprop-P	Propamocarb
Azoxystrobin	Fenpropidin	Propaquizafop
Beflubutamid	Fenpropimorph	Propiconazole
Benalaxyl	Fenpyroximate	Propineb
Benfluralin	Fipronil	Propoxycarbazone
Bensulfuron	Flazasulfuron	Propyzamide
Bentazone	Fluazinam	Prosulfocarb
Benthiavalicarb	Fludioxonil	Prosulfuron
Benzoic acid	Flufenacet (formerly fluthiamide)	Prothioconazole
Beta-Cyfluthrin	Flumioxazin	Pymetrozine
Bifenox	Flupyrsulfuron-methyl (DPX KE 459)	Pyraclostrobin
Boscalid	Fluroxypyr	Pyrimethanil
Bromoxynil	Flusilazole	Pyriproxyfen
Captan	Folpet	Quinoclamine
Carbendazim	Forchlorfenuron	Quinoxyfen
Carfentrazone-ethyl	Formetanate	Quizalofop-P-ethyl
Chloridazon (aka pyrazone)	Fosetyl	Quizalofop-P-tefuryl
Chlormequat (chloride)	Fosthiazate	Rimsulfuron (aka renriduron)
Chlorothalonil	Fuberidazole	S-Metolachlor
	Gibberellin	Silthiofam
		Sodium 5-nitroguaiacolate

Chlorotoluron	Glufosinate	Sodium o-nitrophenolate
Chlorpropham	Glyphosate (incl trimesium aka sulfosate)	Sodium p-nitrophenolate
Chlorpyrifos	Imazalil (aka enilconazole)	Spinosad
Chlorpyrifos-methyl	Imazaquin	Spiroxamine
Chlorsulfuron	Imidacloprid	Sulcotrione
Cinidon ethyl	Indoxacarb	Tebuconazole
Clodinafop	Iodosulfuron-methyl-sodium	Tebufenpyrad
Clofentezine	Fluopicolide	Tepraloxydim
Clomazone	Fluoxastrobin	Tetraconazole
Clopyralid	Ioxynil	Thiabendazole
Clothianidin	Iprodione	Thiacloprid
Copper compounds	Iprovalicarb	Thiamethoxam
Cyazofamid	Isoproturon	Thifensulfuron-methyl
Cyclanilide	Isoxaflutole	Thiophanate-methyl
Cyflufenamid	Lenacil	Thiram
Cyfluthrin	Linuron	Tolclofos-methyl
Cymoxanil	Lufenuron	Tolylfluanid
Cypermethrin	MCPA	Tralkoxydim
Cyprodinil	MCPB	Tri-allate
Cyromazine	Mancozeb	Triadimenol
Deltamethrin	Maneb	Triasulfuron
Desmedipham	Mecoprop	Tribenuron (aka metometuron)
Dicamba	Mecoprop-P	Triclopyr
Difenoconazole	Mepanipyrim	Trifloxystrobin
Diflubenzuron	Mepiquat	Triflusulfuron
Diflufenican	Mesotrione	Trinexapac (aka cimetacarb ethyl)
Dimethachlor	Metamitron	Triticonazole
Dimethenamid-P	Metconazole	Tritosulfuron
Dimethoate	Methiocarb (aka mercaptodimethur)	Ziram
Dimethomorph	Metiram	Zoxamide
Dimoxystrobin	Metrafenone	lambda-Cyhalothrin
Dinocap	Metribuzin	zeta-Cypermethrin
Diuron	Metsulfuron-methyl	
Dodemorph	Milbemectin	
Epoxiconazole	Molinate	
Esfenvalerate		
Ethephon		

The above CAG level 2a for “Delayed development and decreased offspring body weight” comprises different specific effects with varying mode of action and may mainly be useful for general first-step risk assessments. This main CAG can be subdivided into the following level 2 CAGs:

Table 25.3. Overview of CAGs within CAG level 2a “Delayed development and decreased body weight”

Level 2 CAGs within “Decreased offspring body weight and delayed development”	Subgroups, Level 2 CAGs	Specific effects included in the CAG

Delayed development (CAG level 2a1)	Prenatal (CAG level 2a1a)	Delayed ossification Delayed tooth eruption (rabbit) Other (no information on specific effects)
	Postnatal (CAG level 2a1b)	Delayed sexual maturation Delayed eye opening Delayed auditory canal opening Delayed pinna unfolding Delayed tooth eruption (rat, mouse) Other (e.g. no information on specific effects, delayed grip reflex, delayed hair growth)
Decreased body weight (CAG level 2a2)	Prenatal (CAG level 2a2a)	Foetal weight Birth weight
	Postnatal (CAG level 2a2b)	Weight during lactation Weight at weaning
	Adult (CAG level 2a2c)	Weight adult offspring
Increased body weight (CAG level 2a3)	Prenatal (CAG level 2a3a)	Foetal weight
	Postnatal (CAG level 2a3b)	Weight during lactation
	Adult (CAG level 2a3c)	Weight adult offspring

One CAG level 3 is presented within "Delayed development and decreased body weight" for substances that are also known to affect the thyroid hormone system, as this is a likely cause of delayed development and decreased body weight of offspring (see below in section on CAGs at level 3).

25.2.2.2. CAG level 2a1: Delayed development

Appendix AF lists substances with effects on delayed development in offspring. This table collects the information also presented in the Access database in order to give an overview of how one substance can affect more than one endpoint within this main CAG level 2. As delayed development and/or decreased offspring body weights are often seen at doses that also induce maternal toxicity, e.g. decreased body weight of the dams, this is indicated with the letter "t" in the CAG list. More than one line may be presented for the same substance, as a compound may have effects on a certain endpoint (e.g. delayed ossification of foetuses) in one study at a dose at or above the dose causing maternal toxicity, and in another study at a dose for which maternal toxicity is not noted. One such example is Boscalid, see Appendix AF. Another reason why two lines are presented for a compound is when one study describes a specific effect of the parent compound, and another study describes the same effect for a metabolite. One example of this is 2,4 D, see Appendix AF.

The majority of the active substances in the CAG level 2a1 "Delayed development" affect foetal development, whereas fewer active substances affect postnatal development (see Appendix AF). This may in part reflect that postnatal development has not always been examined in detail in one- and/or multi-generation studies, whereas the prenatal development studies always/often include assessment of ossification of foetuses.

Two CAG level 2a1 subgroups CAG level 2a1a “Delayed prenatal development” and CAG level 2a1b “Delayed postnatal development” are established and presented in the following Tables 25.4 and 25.5:

Table 25.4. CAG level 2a1a: Delayed prenatal development (at dose levels with or without maternal toxicity)

2,4-D	Dodemorph	Oxasulfuron
2,4-DB	Ethoxysulfuron	Penconazole
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Etofenprox	Phenmedipham
Abamectin (aka avermectin)	Fenamidone	Phosmet
Amitrole (aminotriazole)	Fenhexamid	Pirimicarb
Azoxystrobin	Fenoxaprop-P	Pirimiphos-methyl
Beflubutamid	Flazasulfuron	Propamocarb
Benalaxyl	Fluazinam	Propaquizafop
Bensulfuron	Flumioxazin	Propoxycarbazone
Bentazone	Fluopicolide	Prothioconazole
Benthiavalicarb	Fluoxastrobin	Pymetrozine
Beta-Cyfluthrin	Fluroxypyr	Pyraclostrobin
Bifenox	Flusilazole	Quinoclamine
Boscalid	Folpet	Quizalofop-P-ethyl
Captan	Forchlorfenuron	S-Metolachlor
Carbendazim	Fuberidazole	Silthiofam
Carfentrazone-ethyl	Glufosinate	Spiroxamine
Chloridazon (aka pyrazone)	Glyphosate (incl trimesium aka sulfosate)	Sulcotrione
Chlorothalonil	Imazalil (aka enilconazole)	Tebuconazole
Chlorotoluron	Imazaquin	Tebufenpyrad
Chlorpropham	Imidacloprid	Tepraloxydim
Chlorpyrifos-methyl	Indoxacarb	Tetraconazole
Chlorsulfuron	Iodosulfuron-methyl-sodium	Thiabendazole
Clodinafop	Ioxynil	Thiacloprid
Clomazone	Isoproturon	Thiamethoxam
Clothianidin	Isoxaflutole	Thifensulfuron-methyl
Copper compounds	Linuron	Thiophanate-methyl
Cyflufenamid	MCPA	Thiram
Cyfluthrin	MCPB	Tolclofos-methyl
Cymoxanil	Mancozeb	Tralkoxydim
Cyprodinil	Maneb	Tri-allate
Cyromazine	Mecoprop	Triadimenol
Deltamethrin	Mecoprop-P	Triasulfuron
Desmedipham	Mesotrione	Tribenuron (aka metometuron)
Dichlorprop-P	Metconazole	Triclopyr
Dimethachlor	Metribuzin	Triflusulfuron
Dinocap	Metsulfuron-methyl	Triticonazole
Diuron	Molinate	Ziram
	Oxadiazon	

Table 25.5. CAG level 2a1b: Delayed postnatal development (at dose levels with or without maternal toxicity)

Amitrole (aminotriazole)	Fluoxastrobin	Propiconazole
Beflubutamid	Fosthiazate	Prothioconazole
Bromoxynil	Glyphosate (incl trimesium aka sulfosate)	Pymetrozine
Chlorothalonil	Iprodione	Pyraclostrobin
Clodinafop	Lufenuron	Spiroxamine
Clothianidin	Mancozeb	Sulcotrione
Cypermethrin	Mecoprop	Tebufenpyrad
Dicamba	Mepiquat	Tepraloxydim
Dimethomorph	Mesotrione	Tetraconazole
Dodemorph	Metconazole	Thiophanate-methyl
Ethofumesate	Metrafenone	Triadimenol
Fenpropidin	Molinate	Trifloxystrobin
Fipronil	Propaquizafop	Tritosulfuron
Fluazinam		

Delayed foetal ossification was observed for all substances shown to delay prenatal development. Delayed ossification should be interpreted in the context of other maternal and foetal findings. In many cases this effect was only seen at dose levels at or above those leading to maternal toxicity (most often reduced body weight and/or food consumption, but also clinical signs, and in some cases abortions or maternal death) and was likely associated with this and it may be argued that these should not be included in a CAG. When leaving out substances for which maternal toxicity is noted, this leaves the following substances in a modified CAG level 2a1a “Delayed prenatal development”:

Table 25.6. Modified CAG level 2a1a: Delayed prenatal development (at dose levels without maternal toxicity)

Abamectin (aka avermectin)	Fluoxastrobin	Metribuzin
Bensulfuron	Flusilazole	Penconazole
Bifenox	Folpet	Phenmedipham
Boscalid	Glyphosate (incl trimesium aka sulfosate)	Prothioconazole
Carbendazim	Imazalil (aka enilconazole)	Tetraconazole
Carfentrazone-ethyl	Ioxynil	Thiacloprid
Cyfluthrin	Isoxaflutole	Thiophanate-methyl
Cymoxanil	MCPA	Tralkoxydim
Deltamethrin	Mesotrione	Triclopyr
Fenoxaprop-P		
Flumioxazin		

The applicability of the modified CAG level 2a1a in Table 25.6 may be discussed as decreased maternal body weight gain may in some cases be secondary to reduced weight of foetuses. Additionally, mild maternal toxicity elicited as decreased food intake, decreased body weight gain or clinical signs may not always be clearly associated with delayed

ossifications, and compounds with these maternal effects may be relevant to include in a CAG for specific effects on skeletal ossification. Importantly, the DARs often use effects on prenatal development, e.g. delayed ossification, for determination of developmental NOAELs also in the presence of mild maternal toxicity (decreased body weight gain, decreased food intake or clinical signs). To use the same principles as applied by the DAR, it would not be appropriate to exclude all compounds having effects at maternal toxic doses. *It is therefore recommended to use the broader CAG level 2a1a presented in Table 25.4 for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded*.

In the case of delayed postnatal development the association to maternal toxicity (e.g. decreased body weight) is not so clear. Before removing the active substances for which the effects are seen at or above maternal toxicity the effect should be checked for e.g. endocrine causes of the delayed development.

A number of compounds (ethofumesate, fipronil, fluazinam, mancozeb, mepiquat, metconazole, pyrimetrozine, tepraloxydim, and trifloxystrobin) all caused delayed development at maternal toxic doses and are removed from Table 25.5 to form the modified CAG level 2a1b below (Table 25.7). The effects caused by these substances were not endocrine related (i.e. they did not affect sexual maturation). Propiconazole and triadimenol also caused delayed development at maternal toxic doses, but as the specific effects of these substances were delayed sexual maturation, which may be considered an endocrine-related effect, these substances are not removed from the modified CAG below. Compounds with endocrine-related effects at doses at or above maternal toxicity levels are therefore included in Table 25.7.

Table 25.7. Modified CAG level 2a1b: Delayed postnatal development (at dose levels without maternal toxicity).

Amitrole (aminotriazole)	Fluoxastrobin	Propiconazole
Beflubutamid	Fosthiazate	Prothioconazole
Bromoxynil	Glyphosate (incl trimesium aka	Pyraclostrobin
Chlorothalonil	sulfosate)	Spiroxamine
Clodinafop	Iprodione	Sulcotrione
Clothianidin	Mecoprop	Tebufenpyrad
Cypermethrin	Mesotrione	Tetraconazole
Dicamba	Metrafenone	Thiophanate-methyl
Dimethomorph	Molinate	Triadimenol
Dodemorph	Propaquizafop	Tritosulfuron
Fenpropidin		

The same line of arguments applies for delayed postnatal development as for delayed prenatal development. It cannot be determined whether mild maternal effects have any causative role in relation to the observed delayed postnatal development, and DARs often use postnatal developmental effects for determination of offspring NOAELs. *It is recommended to use the broader CAG level 2a1b which includes compounds with effects at doses showing mild*

maternal toxicity (Table 25.5) for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded.

The tables below present subgroups of the CAG level 2a1b "Delayed postnatal development" (including effects at dose levels inducing maternal toxicity).

Table 25.8. CAG level 2a1b1: Delayed sexual maturation

Beflubutamid	Glyphosate (incl trimesium aka sulfosate)	Prothioconazole
Chlorothalonil	Iprodione	Pyraclostrobin
Clothianidin	Mesotrione	Spiroxamine
Dicamba	Metrafenone	Tebufenpyrad
Fenpropidin	Molinate	Tetraconazole
Fluoxastrobin	Propiconazole	Triadimenol

Table 25.9. CAG level 2a1b2: Delayed eye opening

Bromoxynil	Lufenuron	Sulcotrione
Clodinafop	Mancozeb	Tepraloxydim
Cypermethrin	Metconazole	Thiophanate-methyl
Dodemorph	Propaquizafop	Trifloxystrobin
Fluazinam	Pymetrozine	Tritosulfuron
Fosthiazate		

Table 25.10. CAG level 2a1b3: Delayed auditory canal opening

Amitrole (aminotriazole)	Dodemorph	Propaquizafop
Cypermethrin	Mecoprop	Tritosulfuron

Table 25.11. CAG level 2a1b4: Delayed pinna unfolding

Clodinafop	Fluazinam	Thiophanate-methyl
Dodemorph	Mecoprop	Tritosulfuron
Fipronil		

Table 25.12. CAG level 2a1b5: Delayed tooth eruption

Clodinafop	Dimethomorph	Fosthiazate

Cypermethrin	Fipronil	

25.2.2.3. CAG level 2a2: Decreased body weight

Appendix AG lists the compounds that have decreased offspring body weights and/or decreased weight gain of offspring prenatally, postnatally (= during weaning) and/or in adulthood. Appendix AG reveals that several active substances decrease both prenatal and postnatal body weight, and that these effects are often seen at maternal toxic dose levels, i.e. doses at which reduced maternal body weight, food consumption and/or other effects are seen.

Table 25.13 presents CAG level 2a2a "Prenatal body weight decrease" and in Table 25.14 this CAG is modified by removing those compounds for which the effects are only seen at maternal toxic doses:

Table 25.13. CAG level 2a2a: Prenatal body weight decrease (including compounds at doses showing maternal effects)

2,4-D	Diuron	Metribuzin
2,4-DB	Dodemorph	Milbemectin
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Esfenvalerate	Molinate
	Ethephon	Oxadiazon
Acetamiprid	Ethofumesate	Oxamyl
Acibenzolar-S-methyl (benzothiadiazole)	Ethoxysulfuron	Oxasulfuron
	Etofenprox	Penconazole
Aclonifen	Fenamidone	Phenmedipham
Amidosulfuron	Fenamiphos (aka phenamiphos)	Phosmet
Amitrole (aminotriazole)	Fenoxaprop-P	Picolinafen
Azimsulfuron	Fenpropimorph	Pirimicarb
Benalaxyl	Flazasulfuron	Propamocarb
Bensulfuron	Fluazinam	Propaquizafop
Bentazone	Flufenacet (formerly fluthiamide)	Propineb
Benzoic acid	Flumioxazin	Propoxycarbazone
Beta-Cyfluthrin	Fluopicolide	Prosulfocarb
Bromoxynil	Flupyrsulfuron-methyl (DPX KE 459)	Prothioconazole
Captan		Pyrimethanil
Carbendazim	Flusilazole	Quinoclamine
Chloridazon (aka pyrazone)	Folpet	Quizalofop-P-tefuryl
Chlorothalonil	Forchlorfenuron	S-Metolachlor
Chlorotoluron	Fosthiazate	Silthiofam
Chlorpropham	Fuberidazole	Spinosad
Chlorpyrifos	Glyphosate (incl trimesium aka sulfosate)	Spiroxamine
Chlorpyrifos-methyl		Sulcotrione
Chlorsulfuron	Imazalil (aka enilconazole)	Tebuconazole
Clodinafop	Imazaquin	Tebufenpyrad
Clofentezine	Imidacloprid	Tepraloxydim
Clomazone	Indoxacarb	Tetraconazole
Clopyralid	Iodosulfuron-methyl-sodium	Thiabendazole
Clothianidin	Ioxynil	Thiacloprid
Copper compounds	Iprodione	Thiamethoxam

Cyflufenamid	Isoproturon	Thifensulfuron-methyl
Cyfluthrin	Isoxaflutole	Thiophanate-methyl
Cymoxanil	Linuron	Thiram
Cyprodinil	MCPA	Tolylfluanid
Cyromazine	MCPB	Tralkoxydim
Deltamethrin	Mancozeb	Tri-allate
Desmedipham	Maneb	Triadimenol
Dichlorprop-P	Mecoprop	Triasulfuron
Difenoconazole	Mecoprop-P	Tribenuron (aka metometuron)
Dimethoate	Mesotrione	Triclopyr
Dimethomorph	Metconazole	Triticonazole
Dinocap	Methiocarb (aka mercaptodimethur)	Ziram
	Metiram	
	Metrafenone	

Table 25.14. Modified CAG level 2a2a: Prenatal body weight decrease (excluding compounds at doses showing maternal effects)

Bentazone	Ethofumesate	Metrafenone
Carbendazim	Fluazinam	Oxamyl
Chlorpropham	Flumioxazin	Penconazole
Chlorpyrifos-methyl?	Fluopicolide	Phenmedipham
Copper compounds?	Fosthiazate	Phosmet
Deltamethrin	Ioxynil	Propamocarb
Desmedipham	Isoproturon	Propineb?
Dichlorprop-P	Isoxaflutole	Quizalofop-P-tefuryl
Difenoconazole?	MCPA	Tepraloxydim
Dimethoate	Mecoprop	Tetraconazole
Dinocap	Mecoprop-P	Thiophanate-methyl
Esfenvalerate	Mesotrione	Thiram?
	Metiram	Triclopyr

? = Not clear from DARs whether maternal effects are observed at same dose levels

Decreases in prenatal body weight may be related to decreases in dam body weight, but the same line of arguments applies for decreased prenatal body weight as for delayed prenatal development. It cannot be determined whether mild maternal effects have any causative role in relation to the observed decreased prenatal body weight, and DARs often use effects on prenatal body weight for determination of developmental NOAELs. *It is recommended to use the broader CAG level 2a2a which includes compounds with effects at doses showing mild maternal toxicity (Table 25.13) for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded.*

Decreases in prenatal body weight may be related to decreases in dam body weight, whereas decreases in postnatal body weight may also be caused by postnatal malnourishment (e.g. impaired lactation or lack of maternal care). The two tables below (Tables 25.15 and 15.16)

present two possible CAGs at level 2a2b “Postnatal body weight decrease”. The CAG level 2a2b1 includes active substances that affect postnatal body weight irrespective of whether prenatal body weight is affected, whereas the CAG level 2a2b2 presents only active substances for which no prenatal body weight (but only postnatal bodyweight) reduction was noted. The CAG level 2a2b1 in Table 25.15 thus includes both prenatal and postnatal causes of the observed low postnatal body weight, and the CAG level 2a2b in Table 25.16 includes only postnatal causes of the observed low postnatal body weight. Both CAGs at level 2a2b include substances having effects at doses showing maternal effects.

Table 25.15. CAG level 2a2b: Postnatal body weight decrease (prenatal and/or postnatal causes of effect)

2,4-D	Esfenvalerate	Metsulfuron-methyl
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Ethephon	Milbemectin
	Ethofumesate	Molinate
Abamectin (aka avermectin)	Ethoprophos	Oxamyl
Acetamiprid	Ethoxysulfuron	Oxasulfuron
Acibenzolar-S-methyl (benzothiadiazole)	Etofenprox	Penconazole
	Etoxazole	Phenmedipham
Amidosulfuron	Famoxadone	Phosmet
Amitrole (aminotriazole)	Fenamidone	Pirimicarb
Azimsulfuron	Fenamiphos (aka phenamiphos)	Propamocarb
Azoxystrobin	Fenhexamid	Propiconazole
Beflubutamid	Fenoxaprop-P	Propoxycarbazone
Benalaxyl	Fenpropidin	Propyzamide
Benfluralin	Fenpropimorph	Prosulfocarb
Bensulfuron	Fenpyroximate	Prosulfuron
Bentazone	Fipronil	Prothioconazole
Benthiavalicarb	Fludioxonil	Pymetrozine
Beta-Cyfluthrin	Fluopicolide	Pyraclostrobin
Bifenox	Fluoxastrobin	Pyrimethanil
Boscalid	Flupyrsulfuron-methyl (DPX KE 459)	Pyriproxyfen
Bromoxynil		Quinoxyfen
Captan	Flusilazole	Quinoclamine
Carbendazim	Folpet	Quizalofop-P-ethyl
Chloridazon (aka pyrazone)	Forchlorfenuron	Quizalofop-P-tefuryl
Chlormequat (chloride)	Formetanate	Rimsulfuron (aka renriduron)
Chlorothalonil	Fosetyl	S-Metolachlor
Chlorotoluron	Gibberellin	Silthiofam
Chlorpropham	Glyphosate (incl trimesium aka sulfosate)	Spinosad
Cinidon ethyl		Spiroxamine
Clodinafop	Imazalil (aka enilconazole)	Sulcotrione
Clofentezine	Imidacloprid	Tebuconazole
Clothianidin	Indoxacarb	Tebufenpyrad
Cyazofamid	Iodosulfuron-methyl-sodium	Tepraloxydim
Cyclanilide	Ioxynil	Tetraconazole
Cyflufenamid	Iprodione	Thiabendazole
Cyfluthrin	Iprovalicarb	Thiacloprid
Cymoxanil	Isoproturon	Thiamethoxam
Cypermethrin	Lenacil	Thiophanate-methyl
Cyprodinil	Linuron	Thiram
Cyromazine	Lufenuron	Tolylfluanid

Deltamethrin	MCPA	Tralkoxydim
Desmedipham	MCPB	Tri-allate
Dicamba	Mancozeb	Triadimenol
Dichlorprop-P	Maneb	Triasulfuron
Difenoconazole	Mecoprop	Tribenuron (aka metometuron)
Diflubenzuron	Mecoprop-P	Triclopyr
Diflufenican	Mepanipyrim	Trifloxystrobin
Dimethachlor	Mepiquat	Trinexapac (aka cimetacarb ethyl)
Dimethenamid-P	Mesotrione	Triticonazole
Dimethoate	Metamitron	Tritosulfuron
Dimoxystrobin	Metconazole	Ziram
Dinocap	Methiocarb (aka	Zoxamide
Diuron	mercaptodimethur)	lambda-Cyhalothrin
Dodemorph	Metiram	zeta-Cypermethrin
Epoxiconazole	Metrafenone	
	Metribuzin	

Table 25.16. CAG level 2a2b: Postnatal body weight decrease without decreased prenatal body weight (postnatal cause of effect, e.g. malnutrition)

Abamectin (aka avermectin)	Dodemorph	Metamitron
Azoxystrobin	Epoxiconazole	Metconazole
Beflubutamid	Esfenvalerate	Metsulfuron-methyl
Benfluralin	Ethoprophos	Oxamyl
Bensulfuron	Etoxazole	Propiconazole
Benthiavalicarb	Famoxadone	Propoxycarbazone
Beta-Cyfluthrin	Fenhexamid	Propyzamide
Bifenox	Fenpropidin	Prosulfuron
Boscalid	Fenpyroximate	Pymetrozine
Captan	Fipronil	Pyraclostrobin
Chloridazon (aka pyrazone)	Fludioxonil	Pyriproxyfen
Chlormequat (chloride)	Fluoxastrobin	Quinoxyfen
Chlorothalonil	Flusilazole	Quizalofop-P-ethyl
Cinidon ethyl	Folpet	Rimsulfuron (aka renriduron)
Clodinafop	Forchlorfenuron	Sulcotrione
Cyazofamid	Formetanate	Tebuconazole
Cyclanilide	Fosetyl	Tepraloxydim
Cyfluthrin	Gibberellin	Thiamethoxam
Cypermethrin	Imazalil (aka enilconazole)	Thiophanate-methyl
Deltamethrin	Imidacloprid	Tribenuron (aka metometuron)
Dicamba	Iprovalicarb	Trifloxystrobin
Diflubenzuron	Lenacil	Trinexapac (aka cimetacarb ethyl)
Diflufenican	Lufenuron	Tritosulfuron
Dimethachlor	Mancozeb	Zoxamide
Dimethenamid-P	Mecoprop	lambda-Cyhalothrin
Dimethoate	Mepanipyrim	zeta-Cypermethrin
Dimoxystrobin	Mepiquat	

The CAG level 2a2b without prenatal causes presented in Table 25.16 has some limitations:

- When active substances with prenatal effects are omitted (Table 25.16) there is a risk of excluding some relevant active substances, e.g. active substances for which the postnatal effect is more marked than the prenatal effect although both effects are statistically significant.
- The opposite problem is also present: this CAG may include some irrelevant active substances, e.g. active substances for which effects would indeed be seen on both pre- and postnatal body weight if the same doses had been applied in both prenatal and postnatal studies.

Therefore, this subdivision of the CAG for postnatal body weight decrease may be of limited usefulness and the broader CAG level 2a2b for postnatal body weight decrease presented in Table 25.15 is recommended for cumulative risk assessment.

A number of active substances also affected body weight in adult offspring. As this was observed in multigeneration studies with continuous dosing of offspring in feed it cannot be determined whether this is caused by pre-/postnatal body weight decreases persisting into adulthood or caused by direct effects of exposure to the active substance in adulthood. These substances are allocated to CAG level 2a2c "Decreased body weight of adult offspring:

Table 25.17. CAG level 2a2c: Decreased body weight of adult offspring

Cyfluthrin	Isoproturon	Sodium 5-nitroguaiacolate
Dimethoate	Isoxaflutole	Sodium o-nitrophenolate
Dodemorph	Mancozeb	Sodium p-nitrophenolate
Esfenvalerate	MCPB	Sulcotrione
Ethephon	Penconazole	Thiabendazole
Etofenprox	Propamocarb	Tolylfluanid
Flumioxazin	Prothioconazole	Triadimenol
Ioxynil		

As those substances that also affect pre- or postnatal offspring body weight are already presented in the above recommended CAGs (Table 25.13 and Table 25.15), and direct effects on body weight are considered non-specific and excluded from this project, *it is not recommend to use the CAG level 2a2c "Decreased body weight of adult offspring" for cumulative risk assessment*.

25.2.2.4. CAG level 2a3: Increased body weight

Exposure to a few of the active substances resulted in increased body weight of the offspring either prenatally or postnatal as presented in Appendix AG and listed in the tables below. Generally, DARs question the toxicological relevance of these effects, and it is not recommended to apply CAGs for increased offspring body weight for cumulative risk assessment.

Table 25.18. CAG level 2a3a: Prenatal body weight increase

Flusilazole
Phenmedipham
Propamocarb
Prothioconazole

Table 25.19. CAG level 2a3b: Postnatal body weight increase

Deltamethrin

Table 25.20. CAG level 2a3c: Increased body weight of adult offspring

Lufenuron

25.2.2.5. CAG level 2b: Malformations and variations

Malformations are defined as structural changes considered detrimental to the animal (may also be lethal) and is usually rare. Variations are defined as structural changes considered to have little or no detrimental effect on the animal. Variations may be transient and may occur relatively frequently in the control population. (ref. OECD TG 414 (Prenatal Developmental Toxicity Study)).

The main CAG level 2b "Malformations and variations" is only subdivided to 2 CAGs at level 2, i.e. CAG level 2b1 "Malformations" and CAG level 2b2 "Variations" although it may be possible to also subdivide into e.g. skeletal malformations, skeletal variations, visceral malformations and visceral variations and "other".

Table 25.21. Overview of CAGs within CAG level 2b "Malformations and Variations"

CAG level 2 within "Malformations and variations"	**Subgroup, CAG level 2**	**CAG includes the following specific effects**
Malformations (CAG level 2b1)	Skeletal malformations (CAG level 2b1a)	E.g. fused ribs, malformed bones
	Cleft palate (CAG level 2b1b)	
	Hydrocephalus (CAG level 2b1c)	
	Excencephaly (CAG level 2b1d)	
	Kidney malformations (CAG level 2b1e)	

	Eye malformations (CAG level 2b1f)	E.g. anopthalmia, microophthalmia
	Heart malformations (CAG level 2b1g)	
Variations (CAG level 2b2)	Skeletal variations	E.g. extra ribs, wavy ribs, fused sternebrae
	Urinary tract variations	E.g. dilated renal pelvis, small renal papillae and distended ureter
	Dilated brain vesicles	
	Heart variations	E.g. dilated heart ventricles
	Eye variations	E.g. corneal opacity
Runts (CAG level 2b3)		Runt defined as animal significantly smaller than siblings

Variations and malformations are often seen at doses inducing maternal toxicity, e.g. decreased body weight of dams, as indicated with the letter "t" in Appendix AH. However, this does not imply that the developmental toxicity effects are unspecific effects of maternal toxicity. Also, the effects on the foetus, especially in cases of malformations, are more severe than the effect on the dam.

Appendix AH presents substances for which malformations and variations are seen. From this table a main CAG for all substances inducing malformations and/or variations was created (Table 25.22) as well as CAGs for substances inducing either malformations (Table 25.23) or variations (Table 25.24). The Access database contains information on the specific types of malformations and variations, and this information has been applied to define some subgroups which are presented in Tables 25.25 to 25.37 below.

Table 25.22. CAG level 2b: Malformations and Variations

2,4-D	Ethofumesate	Picolinafen
2,4-DB	Ethoxysulfuron	Pirimicarb
Abamectin (aka avermectin)	Etofenprox	Pirimiphos-methyl
Acibenzolar-S-methyl (benzothiadiazole)	Etoxazole Fenamiphos (aka phenamiphos)	Propamocarb
Amitrole (aminotriazole)	Fenhexamid	Propaquizafop
Azimsulfuron	Fenoxaprop-P	Propiconazole
Beflubutamid	Fenpropidin	Propineb
Bensulfuron	Fenpropimorph	Prosulfocarb
Benthiavalicarb	Fenpyroximate	Prosulfuron
Benzoic acid	Flazasulfuron	Prothioconazole
Beta-Cyfluthrin	Fluazinam	Pymetrozine
Bifenox	Flufenacet (formerly fluthiamide)	Pyraclostrobin
Bromoxynil	Flumioxazin	Pyrimethanil
Captan	Fluoxastrobin	Pyriproxyfen
Carbendazim	Flusilazole	Quinoclamine
Carfentrazone-ethyl	Folpet	Quizalofop-P-ethyl
Chloridazon (aka pyrazone)	Fosetyl	Quizalofop-P-tefuryl
Chlorothalonil	Glufosinate	Rimsulfuron (aka renriduron)
Chlorotoluron	Glyphosate (incl trimesium aka	S-Metolachlor
		Silthiofam

Chlorpropham	sulfosate)	Sodium hypochlorite
Chlorpyrifos	Imazalil (aka enilconazole)	Spiroxamine
Chlorpyrifos-methyl	Imazaquin	Sulcotrione
Cinidon ethyl	Imidacloprid	Tebuconazole
Clodinafop	Iodosulfuron-methyl-sodium	Tebufenpyrad
Clomazone	Ioxynil	Tepraloxydim
Clopyralid	Isoxaflutole	Tetraconazole
Clothianidin	Linuron	Thiabendazole
Copper compounds	MCPA	Thiacloprid
Cyclanilide	Mancozeb	Thiamethoxam
Cyfluthrin	Maneb	Thifensulfuron-methyl
Cymoxanil	Mecoprop	Thiophanate-methyl
Cyromazine	Mecoprop-P	Thiram
Deltamethrin	Mepanipyrim	Tolylfluanid
Desmedipham	Mesotrione	Tralkoxydim
Dichlorprop-P	Metconazole	Tri-allate
Difenoconazole	Metiram	Triadimenol
Diflufenican	Metribuzin	Triasulfuron
Dimethoate	Metsulfuron-methyl	Tribenuron (aka metometuron)
Dimoxystrobin	Molinate	Triclopyr
Dinocap	Oxamyl	Trifloxystrobin
Diuron	Oxasulfuron	Triflusulfuron
Dodemorph	Penconazole	Triticonazole
Epoxiconazole	Phenmedipham	Tritosulfuron
	Phosmet	Ziram

Table 25.23. CAG level 2b1: Malformations

2,4-D	Fenhexamid	Prosulfocarb
2,4-DB	Fenoxaprop-P	Prosulfuron
Abamectin (aka avermectin)	Fenpropidin	Prothioconazole
Acibenzolar-S-methyl	Fenpropimorph	Pymetrozine
(benzothiadiazole)	Fenpyroximate	Pyraclostrobin
Amitrole (aminotriazole)	Flazasulfuron	Pyrimethanil
Beflubutamid	Fluazinam	Quinoclamine
Benthiavalicarb	Flumioxazin	Quizalofop-P-ethyl
Benzoic acid	Flusilazole	Quizalofop-P-tefuryl
Bromoxynil	Fosetyl	S-Metolachlor
Carbendazim	Glyphosate (incl trimesium aka	Silthiofam
Chlorpropham	sulfosate)	Sodium hypochlorite
Chlorpyrifos	Imazaquin	Spiroxamine
Chlorpyrifos-methyl	Ioxynil	Sulcotrione
Cinidon ethyl	MCPA	Tebuconazole
Clopyralid	Mancozeb	Tepraloxydim
Clothianidin	Maneb	Thiabendazole
Copper compounds	Mecoprop	Thiacloprid
Cyclanilide	Mecoprop-P	Thifensulfuron-methyl
Cymoxanil	Mepanipyrim	Thiophanate-methyl
Cyromazine	Mesotrione	Thiram
Desmedipham	Metconazole	Tolylfluanid
Dichlorprop-P	Metiram	Tralkoxydim
Diflufenican	Metribuzin	Tri-allate

Dimethoate	Metsulfuron-methyl	Triadimenol
Dimoxystrobin	Oxamyl	Tribenuron (aka metometuron)
Dinocap	Penconazole	Triclopyr
Dodemorph	Pirimicarb	Trifloxystrobin
Ethofumesate	Pirimiphos-methyl	Triflusulfuron
	Propaquizafop	Ziram
	Propiconazole	
	Propineb	

Table 25.24. CAG level 2b2: Variations

2,4-D	Epoxiconazole	Molinate
2,4-DB	Ethoxysulfuron	Oxasulfuron
Azimsulfuron	Etofenprox	Penconazole
Bensulfuron	Etoxazole	Phenmedipham
Beta-Cyfluthrin	Fenamiphos (aka phenamiphos)	Phosmet
Bifenox	Fluazinam	Picolinafen
Bromoxynil	Flufenacet (formerly fluthiamide)	Pirimiphos-methyl
Captan	Flumioxazin	Propamocarb
Carfentrazone-ethyl	Fluoxastrobin	Pyriproxyfen
Chloridazon (aka pyrazone)	Flusilazole	Quizalofop-P-ethyl
Chlorothalonil	Folpet	Rimsulfuron (aka renriduron)
Chlorotoluron	Glufosinate	Sulcotrione
Chlorpropham	Imazalil (aka enilconazole)	Tebufenpyrad
Clodinafop	Imidacloprid	Tetraconazole
Clomazone	Iodosulfuron-methyl-sodium	Thiamethoxam
Deltamethrin	Ioxynil	Tralkoxydim
Desmedipham	Isoxaflutole	Triadimenol
Difenoconazole	Linuron	Triasulfuron
Dimoxystrobin	Mancozeb	Trifloxystrobin
Diuron	Mecoprop-P	Triticonazole
Dodemorph	Mesotrione	Tritosulfuron
	Metconazole	

Table 25.25. CAG level 2b1a: Skeletal malformations

2,4-D	Ethofumesate	Prothioconazole
2,4-DB	Fenhexamid	Pymetrozine
Amitrole (aminotriazole)	Fenpropidin	Pyraclostrobin
Bromoxynil	Fenpropimorph	Pyrimethanil
Carbendazim	Flusilazole	Quinoclamine
Chlorpropham	Fosetyl	Quizalofop-P-ethyl
Cinidon ethyl	Ioxynil	Quizalofop-P-tefuryl
Cymoxanil	MCPA	S-Metolachlor
Cyromazine	Maneb	Tebuconazole
Desmedipham	Mepanipyrim	Tepraloxydim
Dimethoate	Metconazole	Thiacloprid
Dimoxystrobin	Metiram	Thiram
Dinocap	Metribuzin	Tralkoxydim

	Pirimicarb Pirimiphos-methyl Propaquizafop Propineb	Triadimenol Triclopyr Trifloxystrobin

Table 25.26. CAG level 2b1b: Cleft palate

Abamectin (aka avermectin) Chlorpyrifos-methyl Cymoxanil Dinocap Fenpropimorph Flusilazole Imazaquin	MCPA Mancozeb Maneb Mecoprop Mecoprop-P Penconazole	Propiconazole Prothioconazole Quizalofop-P-tefuryl Silthiofam Thiabendazole Thiram Triadimenol

Table 25.27. CAG level 2b1c: Hydrocephalus

Carbendazim Cymoxanil Dinocap Ioxynil Maneb Metconazole	Metiram Penconazole Propineb Quizalofop-P-tefuryl Spiroxamine	Thiabendazole Thiophanate-methyl Thiram

Table 25.28. CAG level 2b1d: Exencephaly

Abamectin (aka avermectin) Carbendazim	Chlorpyrifos Cymoxanil Dinocap	Maneb Propineb Thiophanate-methyl

Table 25.29. CAG level 2b1e: Kidney malformations

Beflubutamid Benzoic acid Dinocap Fluazinam	Flusilazole Glyphosate (incl trimesium aka sulfosate) Propineb	Quinoclamine Sulcotrione Tebuconazole Thifensulfuron-methyl Tritosulfuron

Table 25.30. CAG level 2b1f: Eye malformations

Abamectin (aka avermectin) Acibenzolar-S-methyl (benzothiadiazole) Benzoic acid Carbendazim Dodemorph	Fenhexamid Fenpyroximate Ioxynil Penconazole Propineb	Prosulfocarb Prothioconazole Quinoclamine Thiophanate-methyl Triclopyr

Table 25.31. CAG level 2b1g: Heart malformations

Cyclanilide Flazasulfuron	Flumioxazin Glyphosate (incl trimesium aka sulfosate)	Quinoclamine Thiram

Table 25.32. CAG level 2b2a: Skeletal variations

2,4-D 2,4-DB Abamectin (aka avermectin) Acibenzolar-S-methyl (benzothiadiazole) Amitrole (aminotriazole) Azimsulfuron Bensulfuron Benthiavalicarb Beta-Cyfluthrin Bifenox Bromoxynil Captan Carbendazim Carfentrazone-ethyl Chloridazon (aka pyrazone) Chlorothalonil Chlorotoluron Chlorpropham Chlorpyrifos Cyclanilide Cymoxanil Cyromazine Deltamethrin Desmedipham Dichlorprop-P Difenoconazole Diflufenican Dimethoate Dimoxystrobin Dinocap Diuron Dodemorph	Epoxiconazole Ethoxysulfuron Etofenprox Etoxazole Fenamiphos (aka phenamiphos) Fenoxaprop-P Fenpyroximate Flazasulfuron Fluazinam Flufenacet (formerly fluthiamide) Flumioxazin Fluoxastrobin Flusilazole Folpet Fosetyl Glufosinate Glyphosate (incl trimesium aka sulfosate) Imazalil (aka enilconazole) Imidacloprid Ioxynil Isoxaflutole Linuron Mancozeb Mecoprop-P Mesotrione Metconazole Metiram Oxasulfuron Penconazole Phenmedipham	Phosmet Picolinafen Pirimicarb Pirimiphos-methyl Propamocarb Propiconazole Propineb Prosulfocarb Prosulfuron Prothioconazole Pymetrozine Pyraclostrobin Pyrimethanil Pyriproxyfen Quizalofop-P-ethyl Rimsulfuron (aka renriduron) Silthiofam Sulcotrione Tebuconazole Tebufenpyrad Tepraloxydim Tetraconazole Thiacloprid Thiamethoxam Thiophanate-methyl Thiram Tralkoxydim Tri-allate Triadimenol Triasulfuron Triclopyr Trifloxystrobin Triticonazole Tritosulfuron

Table 25.33. CAG level 2b2b: Urinary tract malformations

2,4-DB	Ioxynil	Pyraclostrobin
Clodinafop	Isoxaflutole	Pyriproxyfen
Clomazone	Mesotrione	Quinoclamine
Difenoconazole	Metconazole	Quizalofop-P-tefuryl
Flusilazole	Propamocarb	Thiacloprid
Glufosinate	Propaquizafop	Thifensulfuron-methyl
Iodosulfuron-methyl-sodium	Prosulfocarb	Triticonazole
	Prothioconazole	Tritosulfuron

Table 25.34. CAG level 2b2c: Dilated brain vesicles

Ethoxysulfuron	Mancozeb	Molinate
Fluoxastrobin	Metiram	

Table 25.35. CAG level 2b2d: Heart variations

Cyclanilide	Ethofumesate	Metribuzin
Cymoxanil	Glyphosate (incl trimesium aka sulfosate)	

Table 25.36. CAG level 2b2e: Eye variations

Metconazole	Sulcotrione	

Table 25.37. CAG level 2b3: Runts

Benthiavalicarb	Isoproturon	Pyrimethanil
Flusilazole	Molinate	Tebuconazole
Folpet	Penconazole	Thiram
Imazalil (aka enilconazole)		

Recommendations concerning malformations and variations: The main level 2 CAGs 2b1 Malformations and 2b2 Variations are recommended, but also the subgroups are recommended.

25.2.2.6. CAG level 2c: Pre- and postnatal death

The main CAG level 2c "Pre- and postnatal death" can be subdivided to two CAGs at level 2, i.e. CAG level 2c1 "Prenatal death" and CAG level 2c2 "Postnatal death".

Table 25.38. Overview of CAGs within CAG level 2c "Pre- and postnatal death"

Level 2 CAG	Subgroup, Level 2 CAG	CAG includes the following specific effects
Death of offspring (CAG level 2c)	Prenatal death (CAG level 2c1)	Resorptions Postimplantation loss Abortions Stillborns Decreased litter size (some also in CAG for female fertility or offspring, other effects, see below) Total litter loss
	Postnatal death (CAG level 2c2)	During lactation At weaning

Postnatal death of pups generally covers the lactation period. An active substance affecting litter size can be placed in both the CAG for fertility and the CAG for prenatal death, if there is no clear indication of the cause of the decreased litter size in generation F1 of a multigeneration study. In prenatal developmental studies where dams are dosed after implantation of foetuses, decreased litter size is only categorized as death of foetus, and decreased litter size in generation F2 of a multigeneration study is categorized as "other effects on offspring".

Appendix AI presents level 2 CAGs for pre- and postnatal death. Fetal death is often seen at doses inducing maternal toxicity, e.g. decreased body weight of dams, as indicated with the letter "t" in Appendix AI. However, this does not imply that the developmental toxicity effects are unspecific effects of maternal toxicity. Also, the effect on the offspring, death, is more severe than the effect on the dam. Therefore, no CAGs excluding compounds with effects at doses showing maternal toxicity are presented.

A main CAG for all substances inducing prenatal and/or postnatal death was created (Table 25.39) together with CAGs for substances inducing either prenatal (Table 25.40) or postnatal (Table 25.41) death.

Table 25.39. CAG level 2c: Prenatal and postnatal death

2,4-D	Ethofumesate	Milbemectin
2,4-DB	Ethoprophos	Molinate
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Ethoxysulfuron	Oxadiazon
	Famoxadone	Oxamyl
Abamectin (aka avermectin)	Fenhexamid	Oxasulfuron

Acetamiprid	Fenoxaprop-P	Penconazole
Acibenzolar-S-methyl (benzothiadiazole)	Fenpropimorph	Phenmedipham
	Fipronil	Phosmet
Aclonifen	Flazasulfuron	Picolinafen
Amitrole (aminotriazole)	Fluazinam	Propamocarb
Azimsulfuron	Flumioxazin	Propaquizafop
Benfluralin	Fluopicolide	Propiconazole
Bensulfuron	Flupyrsulfuron-methyl (DPX KE 459)	Propineb
Bentazone		Propoxycarbazone
Benthiavalicarb	Fluroxypyr	Propyzamide
Benzoic acid	Flusilazole	Prosulfocarb
Beta-Cyfluthrin	Folpet	Prothioconazole
Boscalid	Forchlorfenuron	Quinoxyfen
Captan	Formetanate	Pymetrozine
Carbendazim	Fosetyl	Pyraclostrobin
Chloridazon (aka pyrazone)	Fosthiazate	Pyriproxyfen
Chlormequat (chloride)	Fuberidazole	Quinoclamine
Chlorothalonil	Gibberellin	Quizalofop-P-ethyl
Chlorotoluron	Glufosinate	Quizalofop-P-tefuryl
Chlorpropham	Glyphosate (incl trimesium aka sulfosate)	Silthiofam
Chlorpyrifos		Sodium 5-nitroguaiacolate
Clodinafop	Imazalil (aka enilconazole)	Sodium o-nitrophenolate
Clomazone	Imazaquin	Sodium p-nitrophenolate
Clopyralid	Imazosulfuron	Spinosad
Clothianidin	Indoxacarb	Spiroxamine
Copper compounds	Iodosulfuron-methyl-sodium	Sulcotrione
Cyclanilide	Ioxynil	Tebuconazole
Cyfluthrin	Iprodione	Tebufenpyrad
Cymoxanil	Iprovalicarb	Tepraloxydim
Cypermethrin	Isoproturon	Tetraconazole
Cyromazine	Isoxaflutole	Thiabendazole
Deltamethrin	Linuron	Thiacloprid
Desmedipham	Lufenuron	Thiamethoxam
Dicamba	MCPA	Thiophanate-methyl
Dichlorprop-P	Mancozeb	Thiram
Difenoconazole	Maneb	Tolclofos-methyl
Diflufenican	Mecoprop	Tolylfluanid
Dimethenamid-P	Mecoprop-P	Tralkoxydim
Dimethoate	Mepanipyrim	Tri-allate
Dimethomorph	Mepiquat	Triadimenol
Dimoxystrobin	Mesotrione	Tribenuron (aka metometuron)
Dinocap	Metamitron	Triclopyr
Diuron	Metconazole	Triflusulfuron
Dodemorph	Methiocarb (aka mercaptodimethur)	Trinexapac (aka cimetacarb ethyl)
Epoxiconazole		Triticonazole
Esfenvalerate	Metiram	Tritosulfuron
Ethephon	Metrafenone	Ziram
Etofenprox	Metribuzin	lambda-Cyhalothrin
Etoxazole	Metsulfuron-methyl	zeta-Cypermethrin

Table 25.40. CAG level 2c1: Prenatal death

2,4-D	Etofenprox	Oxamyl
2,4-DB	Famoxadone	Oxasulfuron
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Fenhexamid	Penconazole
Abamectin (aka avermectin)	Fenoxaprop-P	Phosmet
Acibenzolar-S-methyl (benzothiadiazole)	Fenpropimorph	Picolinafen
Aclonifen	Fipronil	Propamocarb
Amitrole (aminotriazole)	Flazasulfuron	Propaquizafop
Bensulfuron	Fluazinam	Propiconazole
Bentazone	Flumioxazin	Propineb
Benthiavalicarb	Fluopicolide	Propoxycarbazone
Beta-Cyfluthrin	Flupyrsulfuron-methyl (DPX KE 459)	Propyzamide
Boscalid	Fluroxypyr	Prosulfocarb
Captan	Flusilazole	Prothioconazole
Carbendazim	Folpet	Pymetrozine
Chlormequat (chloride)	Forchlorfenuron	Pyraclostrobin
Chlorothalonil	Fosetyl	Pyriproxyfen
Chlorotoluron	Fosthiazate	Quinoclamine
Chlorpropham	Gibberellin	Quinoxyfen
Chlorpyrifos	Glufosinate	Quizalofop-P-ethyl
Clomazone	Glyphosate (incl trimesium aka sulfosate)	Quizalofop-P-tefuryl
Clopyralid	Imazalil (aka enilconazole)	Silthiofam
Clothianidin	Imazaquin	Sodium 5-nitroguaiacolate
Copper compounds	Indoxacarb	Sodium o-nitrophenolate
Cyclanilide	Iodosulfuron-methyl-sodium	Sodium p-nitrophenolate
Cyfluthrin	Ioxynil	Spinosad
Cymoxanil	Iprodione	Spiroxamine
Cypermethrin	Isoproturon	Tebuconazole
Cyromazine	Isoxaflutole	Tebufenpyrad
Desmedipham	Linuron	Tepraloxydim
Dicamba	Lufenuron	Tetraconazole
Dichlorprop-P	MCPA	Thiabendazole
Difenoconazole	Mancozeb	Thiacloprid
Diflufenican	Maneb	Thiamethoxam
Dimethenamid-P	Mecoprop	Thiophanate-methyl
Dimethoate	Mecoprop-P	Thiram
Dimethomorph	Mepanipyrim	Tolclofos-methyl
Dimoxystrobin	Mepiquat	Tolylfluanid
Dinocap	Mesotrione	Tralkoxydim
Diuron	Metconazole	Triadimenol
Dodemorph	Methiocarb (aka mercaptodimethur)	Tribenuron (aka metometuron)
Epoxiconazole	Metiram	Triclopyr
Esfenvalerate	Metrafenone	Triflusulfuron
Ethephon	Metribuzin	Trinexapac (aka cimetacarb ethyl)
Ethofumesate	Metsulfuron-methyl	Triticonazole
Ethoprophos	Milbemectin	Tritosulfuron
Ethoxysulfuron	Molinate	Ziram
	Oxadiazon	lambda-Cyhalothrin
		zeta-Cypermethrin

Table 25.41. CAG level 2c2: Postnatal death

2,4-D	Etofenprox	Oxasulfuron
2,4-DB	Etoxazole	Phenmedipham
Abamectin (aka avermectin)	Famoxadone	Phosmet
Acetamiprid	Fipronil	Propamocarb
Amitrole (aminotriazole)	Flumioxazin	Propaquizafop
Azimsulfuron	Flusilazole	Propoxycarbazone
Benfluralin	Forchlorfenuron	Prothioconazole
Benzoic acid	Formetanate	Pyriproxyfen
Beta-Cyfluthrin	Fosthiazate	Quizalofop-P-ethyl
Boscalid	Fuberidazole	Quizalofop-P-tefuryl
Captan	Imazalil (aka enilconazole)	Spinosad
Chloridazon (aka pyrazone)	Imazosulfuron	Spiroxamine
Chlorothalonil	Indoxacarb	Sulcotrione
Chlorpyrifos	Iodosulfuron-methyl-sodium	Tebuconazole
Clodinafop	Ioxynil	Tetraconazole
Cyfluthrin	Iprodione	Thiacloprid
Cymoxanil	Iprovalicarb	Thiram
Deltamethrin	Isoxaflutole	Tolylfluanid
Diflufenican	Linuron	Tri-allate
Dimethoate	Mecoprop	Triadimenol
Dinocap	Mecoprop-P	Tribenuron (aka metometuron)
Dodemorph	Mepiquat	Triclopyr
Epoxiconazole	Metamitron	Trinexapac (aka cimetacarb ethyl)
Esfenvalerate	Metiram	Triticonazole
Ethephon	Milbemectin	Tritosulfuron
Ethofumesate	Molinate	zeta-Cypermethrin
Ethoprophos	Oxamyl	
Ethoxysulfuron		

Effects on pre- or postnatal death are often used by DARs for determination of developmental or offspring NOAELs. The two CAGs for prenatal death (Table 25.40) and for postnatal death (Table 25.41) are recommended for cumulative risk assessment.

A CAG level 3 “Coagulation inhibitors” can be presented within the CAG level 2 for foetal death, as coagulation inhibition leading to excess bleeding can induce abortions, which are in the current project included in the CAG for fetal death (see below in section on CAGs at level 3).

25.2.2.7. CAG level 2d: Other effects in offspring

The main CAG level 2d “Other effects in offspring” can be subdivided to four main CAGs at level 2 and a number of subgroups, see Table 25.42.

Table 25.42. Overview of CAGs within CAG level 2d “Other effects in offspring”

CAG level 2 within “Other effects in offspring”	Subgroup, CAG level 2	CAG includes the following specific effects

Male offspring (CAG level 2d1)	Changes in reproductive organs of male offspring (CAG level 2d1a)	Organ weight changes Gross pathology Histolopathology Semen quality Altered anogenital distance
	Impaired fertility of male offspring (CAG level 2d1b)	Decreased fertility (e.g. fertility index and copulation index)
Female offspring (CAG level 2d2)	Changes in reproductive organs of female offspring (CAG level 2d2a)	Organ weight changes Gross pathology Histolopathology
	Impaired fertility of female offspring (CAG level 2d2b)	Decreased fertility (e.g. fertility index)) Disturbed oestrus cyclicity Prolonged gestation Impaired parturition
Male and/or female offspring (CAG level 2d3)	Imparied fertility of male and/or female offspring (CAG level 2d3a)	Decreased fertility (e.g. fertility index and litter size (some also in CAG for prenatal death)) Decreased number of implantations
Effects on non-reproductive organs or endpoints in offspring (CAG level 2d4)	Changes in other organs of offspring (CAG level 2d4a)	Organ weight changes Gross pathology Histopathology
	Changes in non-reproductive endpoints in offspring (CAG level 2d4b)	Clinical signs (e.g. tremor, head tilt, torticollis, bloody nose, polyuria, cyanosis, laboured breathing) Other (e.g. altered food consumption, haematological changes, neurological effects in offspring)

Table 25.42 describes one suggested subdivision of the main CAG level 2d in Table 25.43. This subdivision is based on a grouping of effects on reproductive organs and fertility for males and females separately.

The effects included in CAG level 2d4 “Effects on non-reproductive organs or endpoints in offspring” are diverse and are intended to reflect mainly developmental changes due to the exposure during gestation and lactation. Also non-developmental changes may be included in this subgroup, e.g. target organ effects seen in offspring.

Direct effects on parental fertility or reproductive organs are presented in the section below on “Fertility”. The data on reproductive and fertility effects within CAG level 2d “Other effects in offspring” may need to be combined with data from the CAG for “Fertility” to determine CAG level 3 effects. A CAG level 3 has been made for substances acting via antiandrogenic modes of action within the main CAG for Offspring or the CAG for Fertility (see below in section on CAGs at level 3).

Using the available data from the Access database numerous further subdivisions can be made e.g. substances with common histological effects in reproductive organs or substances with effect on the same reproductive organ. Suggested subgroups of the main CAG level 2d are presented in Appendix AJ and in Table 25.44 to 25.50.

Table 25.43. CAG level 2d: Other effects in offspring

2,4-D	Fenpyroximate	Propaquizafop
2,4-DB	Fipronil	Propiconazole
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Fluazinam	Propineb
	Flumioxazin	Propoxycarbazone
Abamectin (aka avermectin)	Fluoxastrobin	Propyzamide
Acetamiprid	Forchlorfenuron	Prosulfocarb
Amidosulfuron	Fosetyl	Prothioconazole
Amitrole (aminotriazole)	Glyphosate (incl trimesium aka sulfosate)	Pyraclostrobin
Azimsulfuron		Pyrimethanil
Beflubutamid	Imazalil (aka enilconazole)	Pyriproxyfen
Benthiavalicarb	Indoxacarb	Quinoclamine
Beta-Cyfluthrin	Iodosulfuron-methyl-sodium	Quizalofop-P-ethyl
Carbendazim	Ioxynil	Quizalofop-P-tefuryl
Chlormequat (chloride)	Iprodione	S-Metolachlor
Chlorothalonil	Iprovalicarb	Sodium 5-nitroguaiacolate
Chlorotoluron	Isoproturon	Sodium o-nitrophenolate
Chlorpropham	Isoxaflutole	Sodium p-nitrophenolate
Chlorpyrifos	Lenacil	Spinosad
Chlorpyrifos-methyl	Linuron	Spiroxamine
Chlorsulfuron	Lufenuron	Sulcotrione
Clodinafop	Mancozeb	Tebuconazole
Clomazone	Maneb	Tepraloxydim
Clothianidin	Mecoprop	Tetraconazole
Copper compounds	Mepanipyrim	Thiabendazole
Cyfluthrin	Mepiquat	Thiacloprid
Cypermethrin	Mepiquat	Thiamethoxam
Deltamethrin	Metamitron	Thifensulfuron-methyl
Desmedipham	Metconazole	Thiophanate-methyl
Diflubenzuron	Metiram	Thiram
Dimethoate	Metrafenone	Tolylfluanid
Dimoxystrobin	Metribuzin	Tralkoxydim
Dinocap	Metsulfuron-methyl	Tri-allate
Epoxiconazole	Milbemectin	Triadimenol
Esfenvalerate	Molinate	Triclopyr
Ethephon	Oxamyl	Trifloxystrobin
Ethofumesate	Penconazole	Triticonazole
Ethoxysulfuron	Penconazole	Tritosulfuron
Etofenprox	Phenmedipham	Ziram
Fenamidone	Picolinafen	Zoxamide
Fenhexamid	Pirimiphos-methyl	zeta-Cypermethrin
Fenoxaprop-P	Propamocarb	
Fenpropidin		

Table 25.44. CAG level 2d1a: Changes in reproductive organs of male offspring

2,4-DB	Esfenvalerate	Propamocarb
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Ethephon	Propaquizafop
	Ethoxysulfuron	Propiconazole

Amidosulfuron	Fenoxaprop-P	Prosulfocarb
Azimsulfuron	Fenpropidin	Prothioconazole
Chlorothalonil	Fenpyroximate	Quizalofop-P-ethyl
Chlorotoluron	Fipronil	Spinosad
Chlorpyrifos-methyl	Isoproturon	Spiroxamine
Clodinafop	Isoxaflutole	Tebuconazole
Clothianidin	Linuron	Thiacloprid
Cypermethrin	Mepanipyrim	Thiamethoxam
Deltamethrin	Metamitron	Thifensulfuron-methyl
Desmedipham	Metrafenone	Tolylfluanid
Dimethoate	Penconazole	Triadimenol
Epoxiconazole	Pirimiphos-methyl	Ziram

For the purpose of identifying active substances with possible similar modes of action, substances with effects on male reproductive organs in offspring (CAG level 2d1a, Table 25.44) are subgrouped into the following CAGs at level 2 for substances with effects indicating impaired male reproductive development or function (Table 25.45 to 25.48). These subgroups are used further in the section presenting CAGs at level 3.

Table 25.45. CAG level 2d1a1: Decreased weight of male reproductive organs of offspring

Amidosulfuron	Dimethoate	Metamitron
Chlorothalonil	Esfenvalerate	Propamocarb
Chlorotoluron	Fenoxaprop-P	Propaquizafop
Clodinafop	Fipronil	Spiroxamine
Cypermethrin	Isoxaflutole	Thiamethoxam
Deltamethrin	Linuron	Thifensulfuron-methyl
Desmedipham		

Table 25.46. CAG level 2d1a2: Reduced semen quality of offspring

Azimsulfuron	Fenpropidin	Metrafenone
Chlorpyrifos-methyl	Glyphosate (incl trimesium aka sulfosate)	Propamocarb
Clothianidin	Isoproturon	Prothioconazole
Dimethoate	Linuron	Quizalofop-P-ethyl
Epoxiconazole		Thiamethoxam
Esfenvalerate		

Table 25.47. CAG level 2d1a3: Change in anogenital distance of male offspring

Deltamethrin	Epoxiconazole	Propiconazole
Dimethoate	Esfenvalerate	Prothioconazole

	Linuron	Tebuconazole Triadimenol

Three of the compounds in the CAG in 25.47 for level 2d1a3 for change in anogenital distance of male offspring also induce nipple retention in male offspring as listed in CAG level 2d1a4, Table 25.48.

Table 25.48. CAG level 2d1a4: Nipple retention in male offspring

Epoxiconazole	Linuron	Tebuconazole

Table 25.49. CAG level 2d1b: Impaired fertility of male offspring

Flumioxazin Propamocarb	Quizalofop-P-tefuryl	Triclopyr

Table 25.50. CAG level 2d2a: Changes in reproductive organs of female offspring

Carbendazim	Fenoxaprop-P	Prothioconazole
Chlorsulfuron	Fipronil	Quizalofop-P-ethyl
Cypermethrin	Fosetyl	Spinosad
Deltamethrin	Lenacil	Spiroxamine
Desmedipham	Linuron	Tebuconazole
Diflubenzuron	Metconazole	Thiacloprid
Epoxiconazole	Penconazole	Triadimenol
Ethephon	Prosulfocarb	Triticonazole
Ethoxysulfuron		

Table 25.51. CAG level 2d2b: Impaired fertility of female offspring

2,4-D	Metconazole	Quizalofop-P-tefuryl
Amitrole (aminotriazole)	Penconazole	Spinosad
Fluazinam	Propamocarb	Tri-allate
Imazalil (aka enilconazole)	Propiconazole	Triadimenol
Isoproturon	Prothioconazole	

Table 25.52. CAG level 2d3a: Impaired fertility of male and/or female offspring

2,4-D	Mepiquat	Prothioconazole

Amitrole (aminotriazole)	Metamitron	Quinoclamine
Dimethoate	Metconazole	Quizalofop-P-ethyl
Ethofumesate	Metrafenone	Sodium 5-nitroguaiacolate
Fenpropidin	Metribuzin	Sodium o-nitrophenolate
Fipronil	Metsulfuron-methyl	Sodium p-nitrophenolate
Fluazinam	Milbemectin	Spinosad
Flumioxazin	Molinate	Tolylfluanid
Forchlorfenuron	Oxamyl	Tri-allate
Fosetyl	Penconazole	Triadimenol
Imazalil (aka enilconazole)	Propineb	Triticonazole
Isoproturon	Propyzamid	
Linuron		

Table 25.53. CAG level 2d4a: Changes in other organs of offspring

2,4-DB	Fenamidone	Propamocarb
2-Phenylphenol (incl. sodium salt	Fenhexamid	Propaquizafop
orthophenyl phenol)	Fenoxaprop-P	Propiconazole
Acetamiprid	Fenpropidin	Propoxycarbazone
Amidosulfuron	Fluazinam	Propyzamide
Amitrole (aminotriazole)	Fluoxastrobin	Prosulfocarb
Beflubutamid	Indoxacarb	Prothioconazole
Benthiavalicarb	Iodosulfuron-methyl-sodium	Pyraclostrobin
Carbendazim	Ioxynil	Pyriproxyfen
Chlormequat (chloride)	Iprovalicarb	Quinoclamine
Chlorothalonil	Isoproturon	Quizalofop-P-ethyl
Chlorotoluron	Isoxaflutole	S-Metolachlor
Chlorpyrifos	Lenacil	Spiroxamine
Chlorpyrifos-methyl	Linuron	Sulcotrione
Clodinafop	Mancozeb	Tebuconazole
Clomazone	Maneb	Thiophanate-methyl
Clothianidin	Mecoprop	Thiram
Copper compounds	Mepanipyrim	Triadimenol
Cyfluthrin	Metamitron	Trifloxystrobin
Desmedipham	Metiram	Zoxamide
Diflubenzuron	Metrafenone	
Dimoxystrobin	Penconazole	
Etofenprox		

The CAG leve 2d4a “Changes in other organs of offspring” includes various effects and will need further subgrouping before being useful for cumulative risk assessment. It may also be possible to form CAGs for 2d4b “Changes in non-reproductive endpoints in offspring”, but at this covers a rather heterogeneous group of endpoints, CAGs have not been formed. Further information on effects included in CAG 2d4 “Changes in non-reproductive organs or endpoints in offspring” can be found in the “Remarks” column of the Access database and may be applicable for further subgrouping if this is considered relevant.

CAG level 2d4 for changes in non-reproductive organs or endpoints in offspring" and subgroups are not recommended for cumulative risk assessment.

Table 25.54. CAG level 2d4b: Altered sex ratio of offspring

2,4-D Abamectin (aka avermectin) Ethofumesate Iprovalicarb Penconazole	Phenmedipham Propamocarb Propoxycarbazone Tetraconazole Thiacloprid	Thifensulfuron-methyl Tralkoxydim Triadimenol Triclopyr

Altered sex ratio often appears to be a chance finding and does not appear to be related to dosing, and the *CAG 2d4b is not recommended for cumulative risk assessment*.

The CAGs at level 2d including subgroups presented in Tables 25.43 to 25.52 are relevant for cumulative risk assessment at level 2, and are all recommended for cumulative risk assessment. It is not recommended to use CAGs level 2d4 for changes in non-reproductive organs or endpoints in offspring presented in Tables 25.53 and 15.54, for cumulative risk assessment.

25.2.2.8. CAG level 2e: Fertility

The main CAG level 2e "Fertility" can be subdivided to five CAGs at level 2, see Table 25.55 and Appendix AK.

Table 25.55. Overview of CAGs within CAG level 2e "Fertility"

CAG level 2 within "Fertility"	Subgroup, CAG level 2	CAG includes the following specific effects
Male (CAG level 2e1)	Changes in male reproductive organs (CAG level 2e1a)	Organ weight changes Gross pathology Histolopathology Semen quality
	Impaired male fertility (CAG level 2e1b)	Decreased fertility (e.g. fertility index) Decreased number of pregnant unexposed females Altered sexual behaviour
Female (CAG level 2e2)	Changes in female reproductive organs (CAG level 2e2a)	Organ weight changes Gross pathology Histolopathology
	Impaired female fertility (CAG level 2e2b)	Decreased fertility (e.g. fertility index), exposed females (e.g. prenatal developmental studies) Disturbed oestrus cyclicity Decreased number of implantations Prolonged gestation Premature delivery Impaired parturition
Male and/or	Impaired male and/or	Decreased fertility (e.g. fertility index and litter size (some

female (CAG level 2e3)	female fertility (CAG level 2e3a)	also in CAG for prenatal death)) when both males and females are exposed (e.g. one-/multigeneration studies) Preimplantation loss/decreased number of implantations when both males and females are exposed

The effects listed here are all direct effects in males or females, i.e. effects in repeated dose studies and effects on parental animals in generation studies. Effects in the reproductive system of offspring were presented in the section above on the CAG Level 2d "Other effects in offspring".

In addition, various relevant subgroups of the CAG level 2e "Fertility" in Table 25.56 can be proposed, e.g. decreased male reproductive organ weights, decreased male fertility and semen quality, decreased female fertility, increased male reproductive organ weights, decreased female reproductive organ weights, decreased female fertility, increased female reproductive organ weights. Using the available data from the Access database numerous subdivisions can be made, e.g. active substances with common histological effects in reproductive organs or substances with effect on the same reproductive organ. Appendix AL and Table 25.57 to 25.65 present relevant subgroups. When evaluating mode of action of substances the information on reproductive and fertility effects within the CAG level 2e "Fertility" may need to be combined with information from the CAG level 2d "Other effects in offspring", see the section on CAGs at level 3 and level 4 for mode and mechanism of action below.

Table 25.56. CAG level 2e: Fertility

2,4-D	Fenamidone	Oxamyl
2,4-DB	Fenamiphos (aka phenamiphos)	Oxasulfuron
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Fenhexamid	Penconazole
	Fenoxaprop-P	Phenmedipham
Abamectin (aka avermectin)	Fenpropidin	Phosmet
Acetamiprid	Fenpropimorph	Picolinafen
Aclonifen	Fenpyroximate	Propamocarb
Amidosulfuron	Fipronil	Propaquizafop
Amitrole (aminotriazole)	Fluazinam	Propiconazole
Azimsulfuron	Fludioxonil	Propineb
Beflubutamid	Flufenacet (formerly fluthiamide)	Propoxycarbazone
Benalaxyl	Flumioxazin	Propyzamide
Benfluralin	Fluopicolide	Prosulfocarb
Bentazone	Fluoxastrobin	Prosulfuron
Benthiavalicarb	Flupyrsulfuron-methyl (DPX KE 459)	Prothioconazole
Beta-Cyfluthrin		Pymetrozine
Bifenox	Flusilazole	Pyraclostrobin
Bromoxynil	Folpet	Pyrimethanil
Carbendazim	Forchlorfenuron	Pyriproxyfen
Chlormequat (chloride)	Formetanate	Quinoclamine
Chlorothalonil	Fosetyl	Quinoxyfen
Chlorotoluron	Fosthiazate	Quizalofop-P-ethyl
Chlorpyrifos	Fuberidazole	Quizalofop-P-tefuryl
Chlorpyrifos-methyl	Gibberellin	Rimsulfuron (aka renriduron)
Chlorsulfuron	Glufosinate	S-Metolachlor
Cinidon ethyl	Glyphosate (incl trimesium aka	Silthiofam

Clodinafop	sulfosate)	Sodium 5-nitroguaiacolate
Clomazone	Imazalil (aka enilconazole)	Sodium o-nitrophenolate
Clothianidin	Imazaquin	Sodium p-nitrophenolate
Cyazofamid	Imidacloprid	Spinosad
Cyclanilide	Indoxacarb	Spiroxamine
Cyflufenamid	Iodosulfuron-methyl-sodium	Sulcotrione
Cyfluthrin	Ioxynil	Tebuconazole
Cymoxanil	Iprodione	Tebufenpyrad
Cypermethrin	Iprovalicarb	Tepraloxydim
Cyromazine	Isoproturon	Tetraconazole
Deltamethrin	Isoxaflutole	Tetraconazole
Desmedipham	Lenacil	Thiabendazole
Dicamba	Linuron	Thiacloprid
Dichlorprop-P	Lufenuron	Thiamethoxam
Difenoconazole	MCPA	Thiophanate-methyl
Diflubenzuron	MCPB	Thiram
Diflufenican	Mancozeb	Tolclofos-methyl
Dimethoate	Maneb	Tolylfluanid
Dimethomorph	Mepanipyrim	Tralkoxydim
Dimoxystrobin	Mepiquat	Tri-allate
Dinocap	Mesotrione	Triadimenol
Diuron	Metamitron	Triasulfuron
Dodemorph	Metconazole	Tribenuron (aka metometuron)
Epoxiconazole	Methiocarb (aka mercaptodimethur)	Triclopyr
Esfenvalerate	Metiram	Trifloxystrobin
Ethephon	Metrafenone	Triflusulfuron
Ethofumesate	Metribuzin	Trinexapac (aka cimetacarb ethyl)
Ethoprophos	Metribuzin	Triticonazole
Ethoxysulfuron	Milbemectin	Tritosulfuron
Etoxazole	Molinate	Warfarin (aka coumaphene)
Famoxadone	Molinate	Ziram
	Oxadiazon	lambda-Cyhalothrin
		zeta-Cypermethrin

Main subdivisions of the CAG level 2e “Fertility” are presented in Tables 25.57 to 25.61:

Table 25.57. CAG level 2e1a: Changes in male reproductive organs

2,4-D	Ethoxysulfuron	Penconazole
2,4-DB	Etoxazole	Phenmedipham
2-Phenylphenol (incl. sodium salt orthophenyl phenol)	Famoxadone	Phosmet
	Fenamidone	Picolinafen
Abamectin (aka avermectin)	Fenoxaprop-P	Propamocarb
Acetamiprid	Fenpropidin	Propaquizafop
Amidosulfuron	Fenpropimorph	Propineb
Azimsulfuron	Fenpyroximate	Propyzamide
Beflubutamid	Fluazinam	Prosulfocarb
Benalaxyl	Fludioxonil	Prosulfuron
Benfluralin	Flufenacet (formerly fluthiamide)	Prothioconazole
Bentazone	Fluoxastrobin	Pymetrozine
Benthiavalicarb	Flupyrsulfuron-methyl (DPX KE 459)	Quinoclamine
Bromoxynil		Quinoxyfen

Carbendazim	Flusilazole	Quizalofop-P-ethyl
Chlormequat (chloride)	Folpet	Quizalofop-P-tefuryl
Chlorothalonil	Forchlorfenuron	Rimsulfuron (aka renriduron)
Chlorotoluron	Formetanate	Silthiofam
Chlorpyrifos	Fosetyl	Sodium 5-nitroguaiacolate
Chlorsulfuron	Fuberidazole	Sodium o-nitrophenolate
Cinidon ethyl	Gibberellin	Sodium p-nitrophenolate
Clodinafop	Glyphosate (incl trimesium aka	Spinosad
Clothianidin	sulfosate)	Spiroxamine
Cyazofamid	Imazalil (aka enilconazole)	Sulcotrione
Cyclanilide	Imazaquin	Tebuconazole
Cyflufenamid	Imidacloprid	Tebufenpyrad
Cyfluthrin	Indoxacarb	Tepraloxydim
Cymoxanil	Iodosulfuron-methyl-sodium	Tetraconazole
Cypermethrin	Iprodione	Thiabendazole
Cyromazine	Iprovalicarb	Thiacloprid
Deltamethrin	Isoproturon	Thiamethoxam
Desmedipham	Isoxaflutole	Thiophanate-methyl
Dichlorprop-P	Linuron	Thiram
Difenoconazole	Lufenuron	Tolclofos-methyl
Diflubenzuron	MCPA	Tolylfluanid
Diflufenican	MCPB	Tralkoxydim
Dimethoate	Mancozeb	Tri-allate
Dimethomorph	Maneb	Triasulfuron
Dimoxystrobin	Mepiquat	Tribenuron (aka metometuron)
Dinocap	Mesotrione	Triclopyr
Diuron	Metamitron	Trifloxystrobin
Esfenvalerate	Metconazole	Triflusulfuron
Ethephon	Methiocarb (aka mercaptodimethur)	Trinexapac (aka cimetacarb ethyl)
Ethoprophos	Metiram	Triticonazole
	Molinate	Tritosulfuron
	Oxasulfuron	lambda-Cyhalothrin
		zeta-Cypermethrin

Table 25.58. CAG level 2e1b: Impaired male fertility

Abamectin (aka avermectin)	Cypermethrin	lambda-Cyhalothrin
Amitrole (aminotriazole)	Dimethoate	zeta-Cypermethrin
Carbendazim	Thiram	

Table 25.59. CAG level 2e2a: Changes in female reproductive organs

2,4-D	Fenpyroximate	Propoxycarbazone
2,4-DB	Fipronil	Propyzamide
Aclonifen	Fluazinam	Prosulfocarb
Amitrole (aminotriazole)	Flufenacet (formerly fluthiamide)	Prosulfuron
Azimsulfuron	Fluoxastrobin	Prothioconazole
Benfluralin	Flupyrsulfuron-methyl (DPX KE	Pymetrozine

Benthiavalicarb	459)	Pyriproxyfen
Beta-Cyfluthrin	Flusilazole	Quinoclamine
Carbendazim	Fosetyl	Quizalofop-P-ethyl
Chlormequat (chloride)	Fuberidazole	Silthiofam
Chlorothalonil	Gibberellin	Sodium 5-nitroguaiacolate
Clodinafop	Glufosinate	Sodium o-nitrophenolate
Clomazone	Imazalil (aka enilconazole)	Sodium p-nitrophenolate
Clothianidin	Imazaquin	Spinosad
Cyclanilide	Iprodione	Spiroxamine
Cyflufenamid	Isoproturon	Tebuconazole
Cyfluthrin	Isoxaflutole	Tepraloxydim
Cymoxanil	Lenacil	Tetraconazole
Cyromazine	Linuron	Thiacloprid
Deltamethrin	Lufenuron	Thiamethoxam
Desmedipham	Mancozeb	Thiophanate-methyl
Dicamba	Mesotrione	Thiram
Difenoconazole	Metamitron	Tolylfluanid
Diflubenzuron	Metconazole	Tralkoxydim
Diflufenican	Metiram	Tri-allate
Dimethoate	Metribuzin	Triadimenol
Dimoxystrobin	Milbemectin	Tribenuron (aka metometuron)
Dinocap	Molinate	Triclopyr
Ethephon	Molinate	Trifloxystrobin
Ethofumesate	Oxasulfuron	Trinexapac (aka cimetacarb ethyl)
Ethoxysulfuron	Penconazole	Triticonazole
Etoxazole	Phenmedipham	Tritosulfuron
Fenamiphos (aka phenamiphos)	Phosmet	Ziram
Fenpropidin	Propamocarb	zeta-Cypermethrin
Fenpropimorph	Propaquizafop	

Table 25.60. CAG level 2e2b: Impaired female fertility

Amitrole (aminotriazole)	Glufosinate	Rimsulfuron (aka renriduron)
Beflubutamid	Imazalil (aka enilconazole)	S-Metolachlor
Carbendazim	Iprodione	Spinosad
Dichlorprop-P	Mancozeb	Tebuconazole
Difenoconazole	Mepanipyrim	Tetraconazole
Diflufenican	Mesotrione	Thiacloprid
Dimethoate	Metconazole	Thiophanate-methyl
Dinocap	Metrafenone	Thiram
Epoxiconazole	Molinate	Tolclofos-methyl
Fenamiphos (aka phenamiphos)	Oxadiazon	Tralkoxydim
Fenhexamid	Penconazole	Tribenuron (aka metometuron)
Fenpropimorph	Propamocarb	Triclopyr
Fluazinam	Propaquizafop	Triticonazole
Fluopicolide	Propiconazole	Tritosulfuron
Flusilazole	Prothioconazole	lambda-Cyhalothrin
Fosthiazate	Quinoclamine	zeta-Cypermethrin
	Quizalofop-P-ethyl	

Table 25.61. CAG level 2e3a: Impaired male and/or female fertility

Aclonifen	Glufosinate	Propiconazole
Amitrole (aminotriazole)	Glyphosate (incl trimesium aka sulfosate)	Propineb
Beta-Cyfluthrin	Imazalil (aka enilconazole)	Propoxycarbazone
Bifenox	Iodosulfuron-methyl-sodium	Prothioconazole
Chlormequat (chloride)	Ioxynil	Pyraclostrobin
Chlorotoluron	Iprodione	Pyrimethanil
Chlorpyrifos-methyl	Isoproturon	Quizalofop-P-ethyl
Chlorsulfuron	Linuron	Sodium 5-nitroguaiacolate
Clodinafop	Lufenuron	Sodium o-nitrophenolate
Clomazone	Mesotrione	Sodium p-nitrophenolate
Cyclanilide	Metamitron	Spinosad
Cymoxanil	Metconazole	Spiroxamine
Cypermethrin	Methiocarb (aka mercaptodimethur)	Tebuconazole
Cyromazine	Metiram	Tepraloxydim
Dimethoate	Metribuzin	Tetraconazole
Dodemorph	Molinate	Thiabendazole
Esfenvalerate	Oxamyl	Thiacloprid
Ethofumesate	Oxasulfuron	Thiophanate-methyl
Ethoprophos	Penconazole	Thiram
Flumioxazin	Phosmet	Triadimenol
Flusilazole	Propamocarb	Triticonazole
Formetanate		zeta-Cypermethrin
Fosthiazate		

For the purpose of identifying active substances with possible similar modes of action, substances with effects on male reproductive organs (Table 25.57) are subgrouped further into the following CAGs at level 2 for substances with effects indicating impaired male reproductive function (Tables 25.62 to 25.65):

Table 25.62. CAG level 2e1a1: Decreased weight of male reproductive organs" and "Small male reproductive organs

2,4-D	Fenpropidin	Propamocarb
2,4-DB	Fenpropimorph	Prothioconazole
Acetamiprid	Fenpyroximate	Pymetrozine
Azimsulfuron	Flufenacet (formerly fluthiamide)?	Quinoxyfen
Beflubutamid	Fluoxastrobin	Quinoclamine
Bentazone	Folpet	Quizalofop-P-ethyl
Benthiavalicarb	Formetanate?	Quizalofop-P-tefuryl
Bromoxynil	Gibberellin	Silthiofam
Carbendazim	Glyphosate (incl trimesium aka	Sodium 5-nitroguaiacolate
ChlorothalonilChlorotoluron	sulfosate)	Sodium o-nitrophenolate
Chlorpyrifos	Imazalil (aka enilconazole)	Sodium p-nitrophenolate
Cyclanilide	Iprodione	Tebuconazole
Cyflufenamid	Iprovalicarb	Tepraloxydim
Cymoxanil	Isoproturon	Tetraconazole
Cypermethrin	Isoxaflutole	Thiamethoxam
Desmedipham	Linuron	Thiophanate-methyl
Dichlorprop-P	MCPA	Thiram
Difenoconazole	MCPB	Tolclofos-methyl
Dimethoate	Mancozeb	Tralkoxydim
Dimethomorph	Maneb	Tri-allate
Dinocap	Metconazole	Triasulfuron
Esfenvalerate	Molinate	Trifloxystrobin
Ethephon	Oxasulfuron	Triflusulfuron
Ethoxysulfuron	Phenmedipham	Trinexapac (aka cimetacarb ethyl)
Etoxazole	Phosmet	Triticonazole
Famoxadone		lambda-Cyhalothrin
		zeta-Cypermethrin

The CAG level 2e1a1 in Table 15.62 includes both the specific effect “decreased weight of male reproductive organs” and the specific effect “small reproductive organs”.

Table 25.63. CAG level 2e1a2: Decreased weight of male reproductive organs in Hershberger assay

Beta-Cyfluthrin	Cyfluthrin	Linuron
Chlorpyrifos-methyl	Iprodione	

Table 25.64. CAG level 2e1a3: Reduced semen quality

2,4-D	Diuron	Prothioconazole
2,4-DB	Esfenvalerate	Pymetrozine
Abamectin (aka avermectin)	Fluoxastrobin	Quinoclamine
Amidosulfuron	Folpet	Quinoxyfen
Azimsulfuron	Iprodione	Quizalofop-P-ethyl
Carbendazim	Isoproturon	Quizalofop-P-tefuryl

Chlormequat (chloride)	Linuron	Spinosad
Chlorotoluron	MCPA	Sulcotrione
Chlorpyrifos	MCPB	Tebuconazole
Clothianidin	Mancozeb	Tepraloxydim
Cymoxanil	Maneb	Tetraconazole
Cypermethrin	Metconazole	Thiamethoxam
Deltamethrin	Molinate	Thiophanate-methyl
Desmedipham	Oxasulfuron	Thiram
Difenoconazole	Penconazole	Tralkoxydim
Dimethoate	Picolinafen	Triflusulfuron
Dimoxystrobin	Propamocarb	Tritosulfuron
	Propaquizafop	lambda-Cyhalothrin
		zeta-Cypermethrin

Table 25.65. CAG level 2e1a4: Leydig cell hyperplasia

Cyflufenamid	Iprovalicarb	Propaquizafop
Flusilazole	Linuron	Tepraloxydim
Iprodione	Metamitron	Tralkoxydim
	Metconazole	Triflusulfuron

The CAG level 2e1a4 “Leydig cell hyperplasia” is combined with the CAG level 2f3 “Leydig cell tumours”, see next section on Tumours.

The CAGs for fertility at level 2e including subgroups presented in Tables 25.57 to 25.65 are all relevant for cumulative risk assessment at level 2, and are all recommended for cumulative risk assessment.

25.2.2.9. CAG level 2f: Tumours in reproductive organs

As noted elsewhere, genotoxic pesticides are not authorized for use in the EU. Therefore, tumours described in DARs can be considered non-genotoxic. Non-genotoxic tumours in reproductive organs can be related to disruption of the endocrine system. For example, some anti-androgenic chemicals are known to induce Leydig cell tumours. The human relevance of Leydig cell tumours is debated, but nevertheless, the detection of Leydig cell tumours in animal studies are a clear indication of endocrine disrupting effects of a chemical (Cook et al., 1999). Therefore CAGs for Leydig cell tumours are relevant.

Also other tumours may be indicative for a putative endocrine related mode of action. Importantly, tumours in reproductive organs may be closely related to other effects in those organs, and compounds listed in CAGs for tumours are likely to be present also in the CAGs for effects on reproductive organs (see section on Fertility) due to e.g. histological or functional effects.

The main CAG level 2f “Tumours in reproductive organs” can be subdivided, see Table 25.66.

Table 25.66. Overview of CAGs within CAG level 2f "Tumours in reproductive organs"

CAG level 2	Subgroup, CAG level 2	CAG includes the following specific effects
Tumours in reproductive organs (CAG level 2f)	Mammary gland tumour (CAG level 2f1)	Adenocarcinoma
	Ovarian tumour (CAG level 2f2)	Adenoma Granulosa-theca cell blastoma Granulosa-theca cell tumour Luteoma
	Testis tumour (CAG level 2f3)	Leydig cell tumour (interstitial cell adenoma)
	Uterus tumour (CAG level 2f4)	Endometrial polyps Adenoma Adenocarcinoma Mixed Müllerian tumours Sarcoma

As presented in Appendix AM some active substances may induce more than one type of tumours in reproductive organs. A collective CAG for tumours in reproductive organs and CAGs for each tumour type are presented below:

Table 25.67. CAG level 2f: Tumours in reproductive organs (including all subtypes)

Benfluralin	Etoxazole	Propaquizafop
Benthiavalicarb	Flusilazole	Prosulfuron
Chlorpropham	Fuberidazole	Quizalofop-P-tefuryl
Chlorsulfuron	Ioxynil	Thiacloprid
Dimethomorph	Iprodione	Tolylfluanid
Diuron	Iprovalicarb	Tralkoxydim
Epoxiconazole	Lenacil	Tribenuron (aka metometuron)
Ethoprophos	Linuron	Triflusulfuron
Ethoxysulfuron	Pirimicarb	Tritosulfuron
		Ziram

Table 25.68. CAG level 2f1: Mamma tumours

Diuron
Lenacil
Tribenuron (aka metometuron)
Tritosulfuron

Table 25.69. CAG level 2f2: Ovary tumours

Diuron	Linuron	Tolylfluanid
Epoxiconazole	Thiacloprid	Tralkoxydim

Table 25.70. CAG level 2f3: Testis tumours (Leydig cell tumours)

Benfluralin	Etoxazole	Prosulfuron
Chlorpropham	Flusilazole	Quizalofop-P-tefuryl
Chlorsulfuron	Iprodione	Tralkoxydim
Dimethomorph	Linuron	Triflusulfuron
	Propaquizafop	Ziram

Table 25.71. CAG level 2f4: Uterus tumours

Benthiavalicarb	Fuberidazole	Pirimicarb
Epoxiconazole	Ioxynil	Thiacloprid
Ethoprophos	Iprovalicarb	Tolylfluanid
Ethoxysulfuron	Linuron	Tralkoxydim

A CAG at level 2 can be made for active substances inducing Leydig cell hyperplasia (see section on Fertility, Table 25.65) and Leydig cell tumours. The inclusion of Leydig cell hyperplasia in the CAG for Leydig cell tumours adds three more active substances to this CAG (metamitron, metconazole and tepraloxydim).

Table 25.72. Modified CAG 2f3: Leydig cell hyperplasia and/or Leydig cell tumours

Benfluralin	Flusilazole	Propaquizafop
Chlorpropham	Iprodione	Prosulfuron
Chlorsulfuron	Iprovalicarb	Quizalofop-P-tefuryl
Cyflufenamid	Linuron	Tepraloxydim
Dimethomorph	Metamitron	Tralkoxydim
Etoxazole	Metconazole	Triflusulfuron
		Ziram

All the CAGs level 2f for tumours including subgroups (Table 25.67 to 25.71) can be recommended for cumulative risk assessment. The modified CAG 2f3 for Leydig cell hyperplasia and/or Leydig cell tumours (Table 25.72) can also be recommended.

25.2.3. CAGs level 3 and level 4: Mode / mechanism of action

Although genotoxicity is a relevant mechanism for a number of potent developmental and reproductive toxicants, this mechanism is not considered relevant for this project as it is assumed that none of the pesticides authorized in the EU have any genotoxic potential.

In contrast, all modes of action which result in effects on the endocrine system could be important and relevant for the effects seen in reproductive toxicity studies. Certain active substances can interfere with the action of steroidal androgens in foetal life, with irreversible consequences for later life stages.

Examples on mode of actions are:

- Anticoagulants inducing excess bleeding may induce abortions when administered to pregnant dams and developmental effects in offspring
- Inhibition of/interference with the thyroid hormone homeostasis in pregnant dams may lead to delayed development of offspring (reviewed by Boas et al., 2009 and Brucker-Davis et al., 1998)
- Inhibition of/interference with the steroid hormone homeostasis (in foetal life) may lead to altered development of the reproductive system in males and females (reviewed in e.g. Waring and Harris, 2011 and Gray et al., 2006)

  ☼ (anti)androgenic compounds may

  inhibit 5α-reductase, thereby blocking the conversion of testosterone to DHT

  act as androgen receptor antagonists

  decrease androgen levels due to inhibitions of enzymes involved in steroid hormone synthesis, e.g. Star,P450c17, P450scc and others (mechanism of action).

  decrease androgen levels due to increased catabolism of steroid hormones

  ☼ (anti)estrogenic compounds may

  act as oestrogen receptor agonism/antagonists

  inhibit aromatase activity (resulting in decreased oestrogen levels)

  induce aromatase activity (resulting in increased oestrogen levels)

  decrease oestrogen levels due to decreased steroid synthesis or increased catabolism of steroid hormones

These modes of action may lead to changes in reproductive organ weight and histology (including endocrine-related tumours) or alter the function of reproductive organs leading to e.g. impaired fertility. Some of the modes of action mentioned above are not detected in the studies described in the old DARs. The reason is that the applied test guidelines were not aimed at detecting effects on endocrine sensitive endpoints and mechanistic studies on endocrine effects were rarely performed. The open literature shows several examples of endocrine effects of active substances for which the DARs revealed no clear effects on fertility/reproduction.

The CAGs for reproduction/fertility and development as listed in the previous sections are all at level 2. For many endpoints, level 3 (mode of action) or level 4 (mechanism of action) categories are not presented as it is difficult to pinpoint exact modes or mechanisms of action for many of the effects described in the DARs. However, inclusion of data from other target organ systems collected in the current project may contribute with knowledge on mode of action. For example, knowledge on active substance effects on the thyroid hormone system has been compared with knowledge on active substances resulting in delayed development, and for this mode of action (interference with thyroid hormone system) a CAG level 3 has been made.

CAGs at level 3 (mode of action) can be subdivided into the following CAGs at level 4 (mechanism of action) on the basis of *in vitro* effects of these active substances. CAGs at level 3 combine findings in DARs with findings in the open literature. CAGs at level 4 are mainly based on information found in the open literature, as DARs reveal little mechanistic information on these endpoints.

Table 25.73. CAGs at level 3 and 4 for reproductive and developmental toxicity

Level 2 effect	Level 3 – Mode of action	Level 4 – Mechanism of action
Delayed development	Interruption of thyroid hormone homeostasis	Various, see section on thyroid
Developmental effects	Anticoagulants	Inhibition of vitamin K epoxide reductase
Altered reproductive organ weight or fertility in male and females following direct exposure (parental generation or repeated dose studies) or indirect exposure (offspring)	Anti-androgenic	AR antagonists (e.g. determined in reporter gene assays) Steroid synthesis inhibitors
	Estrogenic	Estrogenic *in vitro* (e.g. proliferation assays) Aromatase inducers
	Anti-estrogenic	Anti-estrogenic *in vitro* Aromatase inhibitors Steroid synthesis inhibitors

The OECD "Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption" describes endocrine disrupting effects and is intended as a "tool to support regulatory authorities by helping to interpret assay results and suggesting possible additional studies for reducing uncertainty". This document lists possible modes of action of chemicals and for each type of guideline study (e.g. 28 day study test guideline 407 or 2-generation study test guideline 416) the possible effects related to each mode of action is listed. For anti-androgenic compounds, typical effects in 28 day studies, pubertal male studies, Hershberger studies and 1- or 2-generation studies include decreases in weights of epididymides, prostate and seminal vesicles with coagulating glands, accompanied by histopathological changes in those organs. However, similar effects may also be seen with estrogenic compounds (OECD 2011).

In the current project, active substances inducing a decrease in male reproductive organ weights are included in the CAG for substances with effects that "may be related to" an anti-androgenic mode of action. It should be noted, however, that this grouping does not take into account other mechanistic information, and that the effects may be caused by other modes of action than anti-androgenicity.

For females, the OECD "Guidance Document on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption" describes that changes in uterus or ovary weight are seen with estrogenic as well as anti-androgenic compounds in assays such as 28 day studies, pubertal female studies, uterotrophic assays and 1- or 2-generation studies (OECD 2011). However, changes in uterus or ovary weight are also seen with androgenic or antiandrogenic chemicals, and further mechanistic knowledge may be needed to be able to categorize chemicals with effects on females depending on their mode of action.

CAGs at level 3 for anti-androgenic substances, thyroid hormone active substances and anticoagulants are presented below. No CAGs at level 3 were made for estrogenic or anti-estrogenic substances, although this might have been possible based on the information available. For these mechanism of action CAGs at level 4 were established.

25.2.3.1. CAG level 3a: Anti-androgenic mode of action

A CAG level 3a for active substances with effects that may be related to an anti-androgenic mode of action was established (Table 25.74). This CAG combines several previously presented CAGs and includes active substances which

- Reduce male reproductive organ weights in exposed males (presented within the Fertility CAG, see Table 25.62)
- And/or reduce male reproductive organ weights in offspring of exposed dams (within Offspring CAG, see Table 25.45)
- And/or reduce semen quality in exposed males or male offspring of exposed dams, see Table 25.64 and Table 25.46
- And/or reduce male reproductive organ weights in the Hershberger assay (see Table 25.63)

Table 25.74. CAG level 3a: Anti-androgenic mode of action (see text for explanation)

2,4-D	Esfenvalerate	Propamocarb
2,4-DB	Ethephon	Propaquizafop
Abamectin (aka avermectin)	Ethoxysulfuron	Propiconazole
Acetamiprid	Etoxazole	Prothioconazole
Amidosulfuron	Famoxadone	Pymetrozine
Amitrole (aminotriazole)	Fenoxaprop-P	Quinoclamine
Azimsulfuron	Fenpropidin	Quinoxyfen
Beflubutamid	Fenpropimorph	Quizalofop-P-ethyl
Beta-Cyfluthrin	Fenpyroximate	Quizalofop-P-tefuryl
Bentazone	Fipronil	Silthiofam
Benthiavalicarb	Flufenacet (formerly fluthiamide)?	Sodium 5-nitroguaiacolate

Bromoxynil	Fluoxastrobin	Sodium o-nitrophenolate
Carbendazim	Folpet	Sodium p-nitrophenolate
Chlormequat (chloride)	Formetanate?	Spinosad
Chlorothalonil	Gibberellin	Spiroxamine
Chlorotoluron	Glyphosate (incl trimesium aka	Sulcotrione
Chlorpyrifos	sulfosate)	Tebuconazole
Chlorpyrifos-methyl	Imazalil (aka enilconazole)	Tepraloxydim
Clodinafop	Iprodione	Tetraconazole
Clothianidin	Iprovalicarb	Thiamethoxam
Cyclanilide	Isoproturon	Thifensulfuron-methyl
Cyflufenamid	Isoxaflutole	Thiophanate-methyl
Cyfluthrin	Linuron	Thiram
Cymoxanil	MCPA	Tolclofos-methyl
Cypermethrin	MCPB	Tralkoxydim
Deltamethrin	Mancozeb	Tri-allate
Desmedipham	Maneb	Triadimenol
Dichlorprop-P	Metamitron	Triasulfuron
Difenoconazole	Metconazole	Trifloxystrobin
Dimethoate	Metrafenone	Triflusulfuron
Dimethomorph	Molinate	Trinexapac (aka cimetacarb ethyl)
Dimoxystrobin	Oxasulfuron	Triticonazole
Dinocap	Penconazole	Tritosulfuron
Diuron	Phenmedipham	lambda-Cyhalothrin
Epoxiconazole	Phosmet	zeta-Cypermethrin
	Picolinafen	

? = No information on whether the testes weight is increased or decreased

It may be debated whether active substances inducing a decrease in sperm quality should be included in the CAG for anti-androgenic mode of action, as decreased sperm quality could have many other causes than and if this is decided it may also be relevant to include active substances decreasing male fertility. The data available in the Appendices AP to AX and in the Access database makes it possible to modify the above CAG by including or excluding e.g. semen quality or fertility effects.

The CAG level 3a presented in Table 25.74 is recommended for cumulative risk assessment.

25.2.3.2. CAG level 3b: Interruption of maternal thyroid hormone homeostasis as potential mode of action for observed delayed development of offspring

Sixty active substances were identified as having effects on the thyroid gland as presented in the section on thyroid toxicity. Of these active substances, 41 active substances also affect pre- or postnatal development, and the following CAG level 3b for thyroid interrupting active substances have been made within the CAG level 2 for delayed development:

Table 25.75. CAG level 3b: Interruption of maternal thyroid hormone homeostasis as potential mode of action for observed delayed development of offspring (including compounds having effects at maternal toxic doses)

2,4-D	Fenamidone	Prothioconazole
2,4-DB	Fipronil	Pymetrozin
Amitrole (aminotriazole)	Flumioxazin	Silthiofam
Beflubutamid	Fluoxastrobin	Tepraloxydim
Benalaxyl	Fuberidazole	Tetraconazole
Benthiavalicarb	Imidacloprid	Thiabendazole
Boscalid	Ioxynil	Thiacloprid
Chlorpropham	Isoxaflutole	Thiamethoxam
Clodinafop	Lufenuron	Thiophanate-methyl
Cyflufenamid	Mancozeb	Thiram
Cyprodinil	Maneb	Tribenuron (aka metometuron)
Desmedipham	MCPA	Tritosulfuron
Dinocap	MCPB (metabolized to MCPA)	Ziram
Etofenprox	Metribuzin	

It should be noted that for the above 41 compounds it is not definitive that interruption of thyroid hormone homeostasis is the mode of action for the observed delayed development. For some of the above compounds, effects on delayed development appear at doses affecting maternal body weight or food consumption. Those compounds are excluded in the modified CAG level 3b:

Table 25.76. Modified CAG level 3b for interruption of maternal thyroid hormone homeostasis as possible mode of action for delayed development of offspring (excluding compounds having effects on maternal body weight or food consumption)

Amitrole (aminotriazole)	Fluoxastrobin	Prothioconazole
Beflubutamid	Ioxynil	Tetraconazole
Boscalid	Isoxaflutole	Thiacloprid
Clodinafop	MCPA	Thiophanate-methyl
Flumioxazin	Metribuzin	

As mentioned in the section on delayed development, developmental effects at doses also showing maternal toxicity (effects on body weight or food consumption) are often used by DARs for setting developmental NOAELs. Therefore, to use the same principles as applied by the DAR, it would not be appropriate to exclude compounds having effects at maternal toxic doses.

25.2.3.3. CAG level 3c: Coagulation inhibition as mode of action for developmental effects

Anticoagulants are considered to be teratogenic due to similarities to warfarin which induces prenatal death and malformations in experimental animals as well as in humans. Among the listed substances only one (Bentazone) was found to be a coagulation inhibitor acting via inhibition of the enzyme vitamin K epoxide reductase. Bentazone had developmental effects including prenatal death, delayed prenatal development and decreased prenatal body weight. As other active substances (new or old not included in this project) may also be coagulation

inhibitors, a CAG for coagulation inhibition as a mode of action for developmental effects is recommended.

Table 25.77. CAG level 3c: Coagulation inhibition as mode of action for developmental effects

Bentazone

25.2.3.4. CAGs level 4: Mechanism of action

Based on a search in the open literature, CAGs at level 4 for mechanism of actions could be made (Table 25.78). It should be noted, that this information is mainly based on *in vitro* studies, and that such studies have not been made for all active substances. Therefore, these CAGs cannot be considered complete.

Table 25.78. CAG level 4: *In vitro* mechanism of action

AR antagonism	Aromatase inhibition	Aromatase induction	Estrogenicity	Anti-estrogenicity	Steroid synthesis inhibition

2-phenylphenol Bentazone Beta-cyfluthrin Chlorpropham Chlorpyrifos Cyfluthrin Cypermethrin Cyprodinil Deltametrin Dimethomorph Diuron Epoxiconazole Esfenvalerate Fenhexamid Fipronil Fludioxonil Imazalil Iprodione Isoproturon Linuron Methiocarb Pirimiphos-methyl Propiconazole Pyrimethanil Quinoxyfen Tebuconazole Tolyfluanid Triadimenol (lamda-Cyhalothrin)	Difenoconazole Diuron Epoxiconazole Flusilazole Glyphosate Imazalil Linuron Penconazole Propiconazole Tebuconazole Triadimenol Triflusulfuron	Clodinafop Cypermethrin Iprodion Oxadiazon Pirimicarb Propamocarb Thiacloprid	2-phenylphenol Bentazone Chlorpyrifos Cyfluthrin Cypermethrin Deltamethrin Dodemorph Methiocarb Pendimethalin Pirimiphos-methyl Pyriproxyfen Thiabendazole Tolclofos-methyl Triadimenol Tribenuron-methyl	Captan Chlorsulfuron Cyfluthrin Diuron Epoxiconazole Etofenprox Isoproturon Linuron Mecoprop Methiocarb Pendimethalin Propiconazole Tebuconazole Tolclofos-methyl Triadimenol (metabolite)	Chlorpyrifos Cypermethrin Dimethoate Epoxiconazole Esfenvalerate Flusilazole Glyphosate Iprodione Isoproturon Linuron Molinate Propiconazole Tebuconazole

The information on mechanism of action *in vitro* can be applied for identifying CAGs at level 4 for compounds with specific reproductive effects (level 2) that may be attributed to the listed mechanisms of action.

Table 25.79. CAG level 4a: Substances with effects on male or female fertility or reproductive organs *in vivo* (direct effects or effects in offspring, i.e. presented in Table 25.56 or Tables 25.44 to 25.52) and for which AR antagonism is seen *in vitro*

2-phenylphenol Bentazone Beta-cyfluthrin Chlorpyrifos Cyfluthrin Cypermethrin Deltametrin Dimethomorph	Diuron Epoxiconazole Esfenvalerate Fenhexamid Fipronil Fludioxonil Imazalil Isoproturon Linuron	Methiocarb Pirimiphos-methyl Propiconazole Pyrimethanil Quinoxyfen Tebuconazole Tolyfluanid Triadimenol (lamda-Cyhalothrin)

Twenty-seven of the 29 compounds that are AR antagonists *in vitro*, are also present in CAGs for fertility or effects in reproductive organs of males and/or females (exceptions are Chlorpropham and Cypronidil).

Table 25.80. CAG level 4b: Compounds with *in vivo* effects that may be attributed to antiandrogenicity (i.e. presented in Table 25.74) and for which AR antagonism is seen *in vitro*

Bentazone	Dimethomorph	Isoproturon
Beta-cyfluthrin	Diuron	Linuron
Chlorpyrifos	Epoxiconazole	Propiconazole
Cyfluthrin	Esfenvalerate	Quinoxyfen
Cypermethrin	Fipronil	Tebuconazole
Deltametrin	Imazalil	Triadimenol
	Iprodione	Lamda-cyhalothrin

It is noted that 20 of 29 compounds listed as AR antagonists *in vitro* also have effects on the male reproductive system *in vivo*.

Table 25.81. CAG level 4c: Compounds with *in vivo* effects that may be attributed to antiandrogenicity (i.e. presented in Table 25.74) and for which inhibition of steroid synthesis is seen *in vitro*

Chlorpyrifos	Esfenvalerate	Molinate
Cypermethrin	Glyphosate	Linuron
Dimethoate	Iprodione	Propiconazole
Epoxiconazole	Isoproturon	Tebuconazole

It is noted that 12 of 13 compounds listed as steroid synthesis inhibitors *in vitro* also have effects on the male reproductive system *in vivo*.

Table 25.82. CAG level 4d: Compounds with effects in the female reproductive system *in vivo* (i.e. presented in Table 25.59 or Table 25.50 to 25.52) and for which estrogenic activity and/or aromatase induction is seen *in vitro*

Clodinafop	Iprodion	Pyriproxyfen
Cyfluthrin	Propamocarb	Thiacloprid
Cypermethrin		Triadimenol

Deltamethrin		Tribenuron

Cyfluthrin Cypermethrin Deltamethrin Dodemorph	Iprodion Methiocarb Oxadiazon Propamocarb	Pyriproxyfen Thiabendazole Tolclofos-methyl Triadimenol

Ten out of 21 active substances acting as estrogens or aromatase inducers *in vitro* also affect female fertility or female reproductive organs *in vivo*.

Table 25.83. CAG level 4: Endocrine disruption as mechanism of action for tumours in the reproductive system

Substance	**Ovary**	**Uterus**	**Testis**	**Mamma**	**Mechanism *in vitro***
Benfluralin			x		
Benthiavalicarb		x			
Chlorpropham			x		AR antagonist
Chlorsulfuron			x		Anti-oestrogenic
Dimethomorph			x		AR antagonist
Diuron	x			x	AR antagonist, anti-estrogenic
Epoxiconazole	x				AR antagonist, aromatase inhibitor, anti-estrogenic, steroid synthesis inhibitor
Ethoprophos		x			Anti-estrogenic
Ethoxysulfuron		x			
Ethoxazole			x		
Flusilazole			x		Aromatase inhibitor
Fuberidazole		x			
Ioxynil		x			
Iprodione			x		Steroid synthesis inhibitor, AR antagonist
Iprovalicarb		x			
Lenacil				x	
Linuron	x	x	x		AR antagonist, anti-estrogenic, steroid synthesis inhibitor
Pirimicarb		x			
Propaquizafop			x		
Prosulfuron			x		
Quizalofop-P-tefuryl			x		
Thiacloprid	x	x			Aromatase inducer (in vivo)

Tolylfluanid	x	x			
Tralkoxydim	x	x	x		
Tribenuron (aka metometuron)				x	Oestrogenic
Triflusulfuron			x		Aromatase inhibitor
Tritosulfuron				x	
Ziram			x		

This information can be applied for development of the following CAGs for compounds inducing tumours in the reproductive system that can be attributed to AR antagonism:

Table 25.84. CAG level 4e: Compounds inducing tumours that may be attributed to endocrine disruption

Benfluralin	Ethoxazole	Propaquizafop
Benthiavalicarb	Flusilazole	Prosulfuron
Chlorpropham	Fuberidazole	Quizalofop-P-tefuryl
Chlorsulfuron	Ioxynil	Thiacloprid
Dimethomorph	Iprodione	Tolylfluanid
Diuron	Iprovalicarb	Tralkoxydim
Epoxiconazole	Lenacil	Tribenuron (aka metometuron)
Ethoprophos	Linuron	Triflusulfuron
Ethoxysulfuron	Pirimicarb	Tritosulfuron
		Ziram

Table 25.85. CAG level 4f: Compounds inducing tumours that may be attributed to endocrine disruption and for which AR antagonistic activity is seen *in vitro*

Chlorpropham	Diuron	Ethoprophos
Dimethomorph	Epoxiconazole	Iprodione
		Linuron

Table 25.86. CAG level 4g: Compounds inducing tumours that may be attributed to endocrine disruption and for which steroid synthesis inhibition (including aromatase inhibition) is seen *in vitro*

Epoxiconazole	Iprodione	Thiacloprid
Flusilazole	Linuron	

All of the above CAGs at level 3 and level 4 can be recommended for cumulative risk assessment (Table 25.74 to 25.86).

25.3. Discussion of CAGs for reproductive and developmental toxicity

25.3.1. NOAEL/LOAEL selection and inclusion criteria for CAGs

In the Access database NOAELs and LOAELs can be found for all relevant studies in the DARs and other documents as well as studies from the open literature considered appropriate for this purpose. This implies that no selection of the ”best” NOAEL/LOAEL has been done. The idea is that the lowest NOAEL/LOAEL should be used for cumulative risk assessment (as a worst case scenario), and only if the cumulative risk assessment for a specific CAG level 2 points to a high risk, it may be considered to perform further evaluation of which NOAEL/LOAEL is indeed the most correct. The inclusion of all studies in the database will make it possible to make this refinement of the cumulative risk assessment.

Similarly, we have decided to take a precautious approach when distributing specific effects into the different CAGs. Certain effects, e.g. reduction of litter size, can have different causes and belong to different CAGs, and when the cause cannot be determined the active substance is placed in both CAGs. In the example of litter size reduction, this effect may be caused by decreased implantation (an effect belonging to the CAG for male or female fertility) or by death of fetuses. Therefore, an active substance affecting litter size can be placed in both the CAG for fertility and the CAG for prenatal death, if there is no clear indication of the cause of the decreased litter size (in generation F1 of a multigeneration study, see text above).

Thus, there is a risk of “false positives”, i.e. active substances that are placed in a certain CAG where they do not belong, if the findings leading to the categorization to a CAG are not real but e.g. chance findings. The precautious approach of including also “borderline”-findings is a well-reasoned choice in order to keep the risk of “false negatives” as low as possible. With “false negatives” is meant active substances that are not placed in a certain CAG where they belong. In the cumulative risk assessment a high number of “false negatives” would lead to an underestimation of the actual risk, and this should be avoided. However, it is likely that the applied approach for making CAGs has not been able to include all relevant active substances in all CAGs. This is due to lack of data in DARs for some active substances, and to (inherent and unavoidable) variability in the sensitivity of studies to detect effects on some endpoints. Additionally, it was decided to look for reproductive/developmental effects only for the 196 active substances listed as reproductive/developmental toxicants in the preliminary grouping. Thus, other compounds than the 196 active substances listed as reproductive/developmental toxicants could have effects that were not detected using the current approach (false negatives). Overall, we believe that most active substances having major effects according to DARs are also presented in the listed CAGs. A complete and accurate database of active substance effects is not possible with the available data material, but our approach has been able to accommodate the aim of collecting as much information as possible to reach the best possible CAGs.

25.3.2. Mode and mechanism of action for reproductive/developmental toxicity

The knowledge on mode or mechanism of action is limited for some effects on reproduction and development. For some endocrine disrupting effects, there is mechanistic knowledge, but mechanistic studies have not been performed on most of the examined active substances. Therefore, the usefulness of CAGs at level 3 and level 4 within reproductive/developmental toxicity will probably be improved with time and increased knowledge on mode/mechanism of action. Although mechanistic knowledge can make a major contribution to the understanding of mixture effects, it is still important to acknowledge that the *in vivo* effects still are important. This was also the conclusion of an expert mixture workshop in Denmark in 2009 summarizing this as: "A pre-occupation with mechanisms or modes of action as the starting point for the grouping of endocrine disrupters into classes to be subjected to mixtures risk assessment was seen as not practical and scientifically hard to justify. Instead, grouping criteria should focus on common health related effects and the likelihood of co-exposures" (Kortenkamp et al., 2009).

A comparison of *in vitro* findings with in vivo findings revealed that most of the active substances listed in the CAG level 4 as AR antagonists *in vitro* are also present in the CAGs at level 2 for effects on fertility or reproductive organs (direct effects or offspring effects). Exceptions were chlorpropham and cyprodinil. Chlorpropham induced Leydig cell tumors (related to endocrine disruption), but induced no other effects related to fertility or endocrine disruption. Cyprodinil is an AR antagonist but does not reduce reproductive organ weights or fertility in vivo in the studies listed in DARs.

In some cases studies in the open literature revealed fertility effects that were not found in studies presented in DARs, indicating that other study designs than those applied in the guideline studies may reveal relevant effects related to endocrine disruption. For example, reproductive effects of the AR antagonist chlorpyrifos were not found in the DAR but reproductive/fertility effects are described in several studies in open literature.

It is highly interesting that most AR antagonists are recognized by one of various types of effects on the female or male reproductive system. The variability in the types of reproductive effects described in DARs for active substances identified as endocrine disrupters *in vitro* may have several reasons. It is possible that for some compounds the AR antagonizing effect is influenced by competing or synergistic endocrine influences leading do differences in effects. Additionally, it is noteworthy that the guidelines applied in most studies described by DARs do not cover endocrine-sensitive endpoints such as changes in anogenital distance or nipple retention, and that targeted mechanistic analyses of endocrine effects have not been performed.

For other mechanisms of actions than AR antagonism, results showed that some compounds that are aromatase inducers or estrogenic in vivo did not affect male or female reproductive organs or fertility *in vivo* (exposed animals and/or offspring of exposed dams) according to studies presented in the DAR. This may either indicate that not all estrogenic compounds have adverse effects in vivo or that there is a limited sensitivity of guidelines applied in most studies described by DARs to endocrine-related (and particularly estrogenic) effects.

25.3.3. Mode of action for tumours

Various modes of action can be related to Leydig cell hyperplasia and Leydig cell tumours including anti-androgenic modes of action (androgen receptor antagonism, inhibition of 5a-reductase, inhibition of testosterone biosynthesis, aromatase inhibition) but also stimulatory modes of action (agonism of estrogen, gonadotropin releasing hormone (GnRH), and dopamine receptors) (Cook et al., 1999). Most of the active substances in the modified CAG level 2f3 for Leydig cell hyperplasia and tumours are also present in the CAG level 3a for anti-androgenic mode of action *in vivo*. The exceptions are benfluralin, chlorpropham, chlorsulfuron, flusilazole, prosulfuron and ziram. Five of these compounds (exception is chlorpropham) all affect female reproductive organs or female fertility, and benfluralin, chlorsulfuron and ziram increase testis weight in rodent studies. This may demonstrate that although Leydig cell tumors as such may not be relevant to humans (Cook et al., 1999), the ability of compounds to induce Leydig cell tumors appears to be indicative of an endocrine disrupting mode of action.

For e.g. thiacloprid it is noted in DARs that hepatic induction of the enzyme aromatase converting testosterone to estradiol may be the cause of uterine and ovarian tumors due to excess estrogen levels. For compounds acting as steroid synthesis inhibitors, AR antagonists or aromatase inhibitors, a possible mechanism of tumor induction appears to be increased levels of gonadotropins LH and FSH leading to growth induction. It should be noted that modes of action have not been examined for most compounds. Therefore, it appears appropriate to include all compounds with similar effects in CAGs rather than to make separate CAGs for compounds with known mode of action (e.g. aromatase inducers or AR antagonists). A further elaboration can be suggested where CAGs for tumors in reproductive organs may be combined with compounds inducing hyperplasia in reproductive organs, as hyperplasia and tumors may be caused by the same mechanism of action and may have cumulative effects.

Overall, the *in vitro* findings for all listed mechanisms of action appear to be predictive for in vivo effects on reproduction and fertility, although exceptions are noted but cannot be explained at present.

25.4. Recommended CAGs for reproductive and developmental toxicity

The following *CAGs at level 2* are recommended for CRA for reproductive and developmental toxicity:

Delayed prenatal development: It is recommended to use the broader CAG level 2a1a presented in Table 25.4 for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded.

Delayed postnatal development: It is recommended to use the broader CAG level 2a1b which includes compounds with effects at doses showing mild maternal toxicity (Table 25.5) for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded.

Prenatal body weight decrease: It is recommended to use the broader CAG level 2a2a which includes compounds with effects at doses showing mild maternal toxicity (Table 25.13) for cumulative risk assessment. As a refinement of this CAG, only compounds with marked maternal toxicity such as maternal body weight loss during gestation or maternal death may be excluded.

Postnatal body weight decrease: The broader CAG level 2a2b for postnatal body weight decrease presented in Table 25.15 is recommended for cumulative risk assessment.

Decreased body weight of adult offspring: It is **not** recommend to use the CAG level 2a2c "Decreased body weight in adult offspring" for cumulative risk assessment.

Increased body weight: It is **not** recommended to apply CAGs for increased offspring body weight for cumulative risk assessment.

Malformations and variations: The main level 2 CAGs 2b1 Malformations and 2b2 Variations are recommended, but also the subgroups 2b1a to 2b1g are recommended (Tables 25.22 to 25.31).

Pre- and postnatal death: The two CAGs level 2c1 for prenatal death (Table 25.40) and level 2c2 for postnatal death (Table 25.41) are recommended for cumulative risk assessment.

Other effects in offspring: The CAGs at level 2d including subgroups presented in Tables 25.43 to 25.52 are relevant for cumulative risk assessment at level 2, and are all recommended for cumulative risk assessment. It is **not** recommended to use CAGs level 2d4 for changes in non-reproductive organs or endpoints in offspring presented in Tables 25.53 and 25.54, for cumulative risk assessment.

The CAGs at level 2d including subgroups presented in Tables 25.43 to 25.52 are relevant for cumulative risk assessment at level 2, and are all recommended for cumulative risk assessment. It is **not** recommended to use CAGs level 2d4 for changes in non-reproductive organs or endpoints in offspring presented in Tables 25.53 and 25.54, for cumulative risk assessment.

All the CAGs level 2f for tumours including subgroups (Table 25.67 to 25.71) can be recommended for cumulative risk assessment. The modified CAG 2f3 for Leydig cell hyperplasia and/or Leydig cell tumours (Table 25.72) can also be recommended.

The following *CAGs at level 3 and 4* are recommended for CRA for reproductive and developmental toxicity:

All of the listed CAGs at level 3 and level 4 (Tables 25.74 to 25.86) can be recommended for CRA.

26. Salivary glands

26.1. Introduction

The salivary glands produce saliva and the enzyme amylase to aid in digestion. The three main types of salivary glands in humans are the parotid, the submandibular and the sublingual salivary glands – see Figure 26.1. Acinar cells are responsible for the secretions by the salivary glands. Salivary gland secretion is controlled by the autonomic nervous system via adrenergic receptors on e.g. the acinar cells.

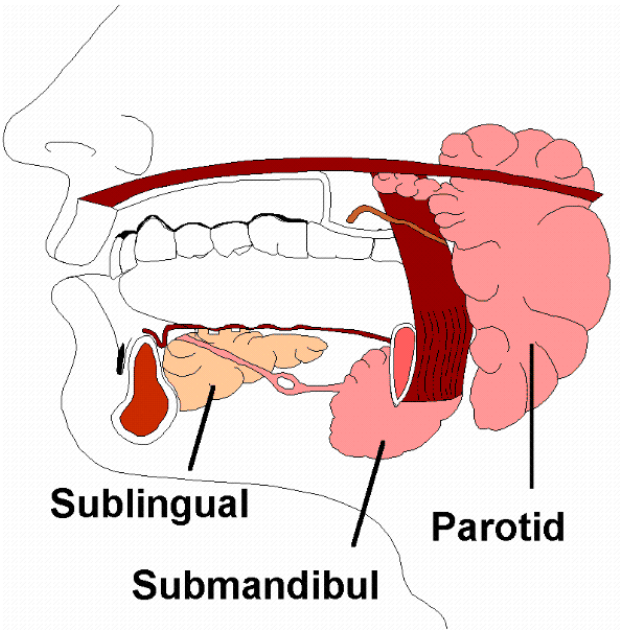


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Figure 26.1. Anatomy of the salivary glands

26.2. Establishment of CAGs for toxicity to the salivary glands

Only a few active substances were identified to affect the salivary glands. The predominant effects reported include hypertrophy and vacuoles. The effects were generally observed only in one study for a particular active substance and the findings were often considered in the DARs not to be treatment-related. Thus, the salivary glands seem not to be primary target organs for the active substances included in this project.

Overall, CRA for effects on the salivary glands are not considered relevant. Therefore, the salivary glands are not considered further for CAGs in this project.

26.3. Recommended CAGs for the salivary glands

No CAGs for toxicity to the salivary glands are recommended.

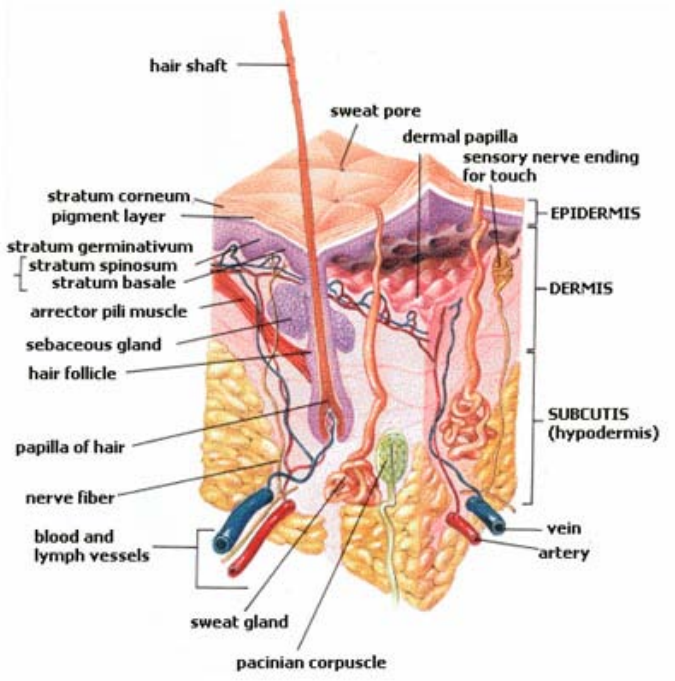
27. Skin

27.1. Introduction

The skin is the outer covering of the body. The skin is necessary to protect the body against pathogens and excessive water loss. The skin is also important in the regulation of temperature and in vitamin D synthesis. The skin contains a variety of nerve endings that allows us to sense the world when they react to heat and cold, touch, pressure, vibration, and tissue injury.

The skin is composed of three primary layers (Figure 27.1):

- The epidermis
- The dermis
- The hypodermis



From http://www.web-books.com/eLibrary/Medicine/Physiology/Skin/Skin.htm

Figure 27.1. Anatomy of the skin

The *epidermis* provides waterproofing and serves as a barrier to infection. The epidermis is further subdivided. The stratum corneum is the outer layer of the epidermis. The epidermis contains no blood vessels. Keratinocytes, which are the predominant cell type in the epidermis, are formed at the inner layer of the epidermis and move up the layers changing shape and composition as they die due to isolation from their blood source. The cytoplasm is released and the protein keratin is inserted. They eventually reach the stratum corneum and slough off.

The *dermis* is the layer of skin beneath the epidermis that consists of connective tissue, nerve endings, blood vessels, lymphatic vessels, hair follicles, and sweat and sebaceous glands.

The *hypodermis* lies below the dermis. Its purpose is to attach the skin to underlying bones and muscles as well as supplying it with blood vessels and nerves. The hypodermis contains 50% of body fat which serves as padding and insulation for the body.

27.2. Establishment of CAGs for toxicity to the skin

Only a few active substances were identified to affect the skin. The predominant effects reported include local dermal effects. The effects were generally observed only in one study for a particular active substance. It is plausible that the effects reported are caused by local irritant properties of the active substances and due to that the active substances in the diet may come in direct contact with the skin rather than caused following systemic uptake of the substance.

Overall, CRA for effects on the skin are not considered relevant. Therefore, the skin is not considered further for CAGs in this project.

27.3. Recommended CAGs for the skin

No CAGs for toxicity to the skin are recommended.

28. Spleen

28.1. Introduction

The spleen is a concave, encapsulated organ, which is located in the left upper abdominal cavity, curved around a portion of the stomach. Strands of connective tissue (trabeculae) extend throughout the spleen from the splenic capsule, dividing the spleen into compartments. The compartments contain masses of lymphoid tissue called splenic pulp. The spleen is the largest of the secondary lymphoid organs.

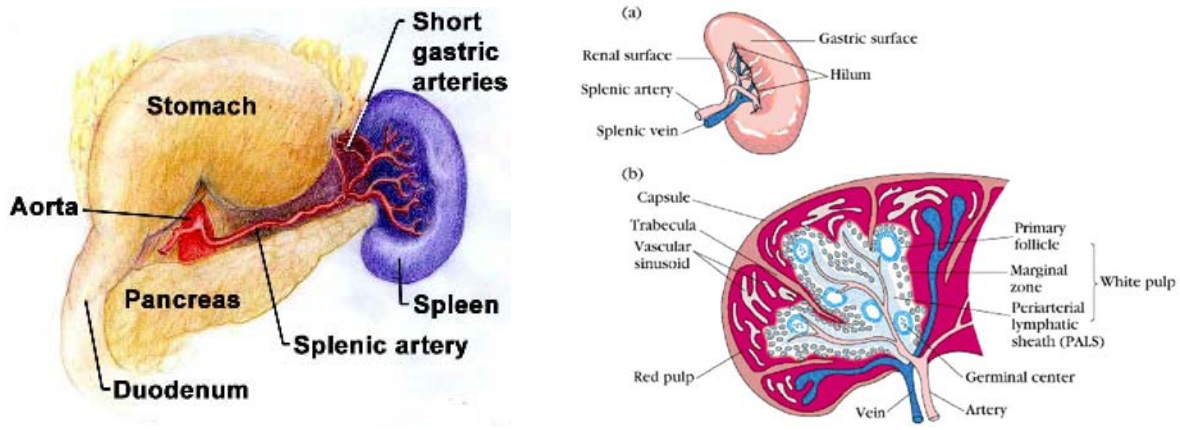


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Figure 28.1. Location and anatomy of the spleen

The portion of arterial blood that enters the spleen first encounters the white splenic pulp, which consists of masses of lymphoid tissue containing lymphocytes and macrophages and is the chief site of immune and phagocytic function within the spleen. Here blood-borne antigens encounter lymphocytes, initiating the immune response, see also Chapter 15.

Most of the blood oozes through the extremely permeable capillary walls into the principal site of splenic filtration, the red pulp. Here the resident macrophages phagocytose old, damaged, or dead blood cells of all kinds (but chiefly red blood cells), micro-organisms, and particles of debris. Haemoglobin from phagocytosed red blood cells is catabolised, and heme (iron) is stored in the cytoplasm of the macrophages or released back into the blood plasma. Blood that filters through the red pulp also finds its way into the venous sinuses, highly distensible storage areas which serves as a blood reservoir, and hence into the portal circulation.

Other functions of the spleen are less prominent, especially in the healthy adult, and include production of red blood cells. While the bone marrow is the primary site of haematopoiesis in the adult, the spleen has important haematopoietic functions up until the fifth month of gestation. After birth, erythropoietic functions cease, except in some haematological disorders. As a major lymphoid organ and a central player in the reticuloendothelial system, the spleen retains the ability to produce lymphocytes and, as such, remains an haematopoietic organ. This is called extramedullary haematopoiesis.

28.2. Establishment of CAGs for toxicity to the spleen

28.2.1. CAG level 1: Toxicity to the spleen

Various types of effects on the spleen were identified including:

- Hyperplasia
- Hypertrophy
- Hypercellularity
- Increased extramedullary haematopoiesis
- Haemosiderosis (increased amounts of a yellow-brown iron containing pigment)
- (Lymphoid) hypoplasia
- Lymphoid depletion
- Reduced cellularity
- Decreased haematopoiesis
- Atrophy
- Necrosis
- Congestion

Most of the active substances identified to affect the spleen induced hyperplasia, hypertrophy, hypercellularity, increased extramedullary haematopoiesis, haemosiderosis, effects that are secondary to a direct effect on the cellular elements in the blood stream and are therefore covered by the CAGs for the cellular elements in the blood, see Chapter 14. Overall, these effects are not considered relevant in terms of CRA for direct effects on the spleen and the active substances causing these effects are therefore not considered further for CAGs in relation to the spleen.

Some of the active substances identified to affect the spleen induced reduced hypocellurarity, hypoplasia, lymphoid depletion, decreased haematopoiesis, atrophy and/or necrosis, effects that are considered as a direct effect on the spleen. The active substances identified as causing these effects in the spleen in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 28.1.

Table 28.1. CAG level 1: Toxicity to the spleen

2,4-D	Clodinafop	MCPA
Acibenzolar-S-methyl	Clothianidin	Metconazole
Acibenzolar-S-methyl metabolite: CGA 210007	Cymoxanil	Oxasulfuron
Bifenazate	Florasulam	Thiamethoxam
Chlorotoluron	Fluroxypyr	Thiophanate-methyl
Chlorpropham	Flutolanil	
Chlorsulfuron metabolite: IN-A4097	Indoxacarb	

28.2.2. CAG level 2: Phenomenological / specific effects on the spleen

Based on the effects on the spleen considered as being direct effects, two distinct CAGs level 2 are proposed. More information is given in Appendix AN.

28.2.2.1. CAG level 2a: Hypoplasia

Hypoplasia is a decrease in the number of cells.

For the purpose of the CAG project, hypoplasia, reduced cellularity, lymphoid depletion and decreased haematopoiesis in the spleen are allocated to a single CAG level 2, termed 'CAG level 2a: Hypoplasia'.

The active substances identified as inducing one of the abovementioned effects in the spleen are allocated to CAG level 2a and listed in Table 28.2.

Table 28.2. CAG level 2a: Hypoplasia

2,4-D	Florasulam	Metconazole
Bifenazate	Fluroxypyr	Oxasulfuron
Clothianidin	Indoxacarb	Thiophanate-methyl

28.2.2.2. CAG level 2b: Cell degeneration / cell death

Atrophy is loss of tissues, totally or partially and necrosis is death of cells and tissues.

For the purpose of the CAG project, histopathological findings described as atrophy and necrosis are allocated to a single CAG level 2, termed 'CAG level 2b: 'Cell degeneration / cell death'.

The active substances identified as inducing one or more of the above-mentioned effects in the spleen are allocated to CAG level 2b and are listed in Table 28.3.

Table 28.3. CAG level 2b: Cell degeneration / cell death

2,4-D	Chlorsulfuron metabolite: IN-A4097	Indoxacarb
Acibenzolar-S-methyl	Clodinafop	MCPA
Acibenzolar-S-methyl metabolite: CGA 210007	Clothianidin	Metconazole
Bifenazate	Cymoxanil	Thiamethoxam
Chlorotoluron	Flutolanil	Thiophanate-methyl
Chlorpropham		

28.2.2.3. Effects not considered relevant for CAGs at level 2

Effects such as discolouration and congestion of the spleen have been reported for some substances. These effects are considered as being non-adverse or non-specific effects and therefore, not relevant for CAGs at level 2 and consequently, not relevant in terms of CRA for effects on the spleen.

28.2.3. CAG level 3: Mode of action

No information on mode of action has been found for any of the active substances considered as having a direct effect on the spleen.

28.2.4. CAG level 4: Mechanism of action

No information on mechanism(s) of action has been found for any of the active substances considered as having a direct effect on the spleen.

28.3. Discussion of CAGs for the spleen

Nineteen active substances or metabolites were identified to have potential direct effects on the spleen and were allocated to CAG level 1. Two distinct CAGs at level 2 have been proposed. Information on mode / mechanism(s) of action is not available for any of them. The information is summarised in Appendix AO.

As no information regarding the mode / mechanism(s) of action for the active substances allocated to CAG level 2a and 2b, respectively, has been found, these CAGs at level 2 could be considered for CRA.

28.4. Recommended CAGs for the spleen

The following CAGs at level 2 are recommended for CRA for effects on the spleen:

- CAG level 2a: Hypoplasia, see Table 28.2.
- CAG level 2b: Cell degeneration / cell death, see Table 28.3.

29. Thymus

29.1. Introduction

The thymus is one of the primary lymphoid tissues consisting of two independent lobes (cortex and medulla) attached to each other by connective tissue. The two independent lobes are comprised by smaller lobules, which basically have the same architecture (a sub-capsular and outer cortical area, a cortex, and a medulla). A unique feature of the thymus compared to other lymphoid organs is that its microenvironment consists of a reticular epithelium.

The basic architecture of the thymus depends on the age and stress hormone status of the individual and a 'normal' architecture can only be expected between the late gestational period and young adulthood. The organ is fully developed around day 17 of gestation in rodents and at 16-17th week of gestation in humans. The first contact with exogenous antigens after birth directs significant thymus growth in a relatively short time as it has to provide large numbers of T cells (lymphocytes produced in the bone marrow before entering the blood and passing through the thymus) to secondary lymphoid tissues. When adulthood is reached, the organ starts to involute (atrophy) and emigration of T cells decreases dramatically. Acute stress or toxic compounds may cause artificial thymus involution leading to a decrease in emigrating T cells.

T cells mature and obtain specificity, from progenitor cells to antigen reactive cells, in their passage through the thymus. T cell maturation occurs in different microenvironments; the least mature cells enter the lobules from the bloodstream at the cortico-medullary junction and then move to the outer sub-capsular cortex (large lymphoblasts) before passing through the cortex (small lymphocytes) and migrating to the medulla (mature T cells). Most of the cells that enter the thymus die due to a process called 'negative selection' which is designed to eliminate cells with the potential to react in a damaging way with self antigens.

29.2. Establishment of CAGs for toxicity to the thymus

Many active substances were identified to affect the thymus. The predominant effect reported is decreased thymus weight. Atrophy (thymic involution) has also been reported for several of the substances. In general, the overall study NOAELs and LOAELs are lower than for the specific 'thymus NOAEL' and 'thymus LOAEL' for the majority of the active substances. Stress, which in many cases is characterised by increased adrenal weight (and increased levels of cortisole) is known to induce decreased thymus weight, which is thus an indirect effect on the thymus. Thus, the thymus seems not to be a primary target organ for the active substances included in this project. Furthermore, changes in thymus weight or atrophy were reported primarily in association with marked effects for other organs, especially in the liver.

Overall, CRA for effects on the thymus is not considered relevant. Therefore, the thymus is not considered further for CAGs in this project.

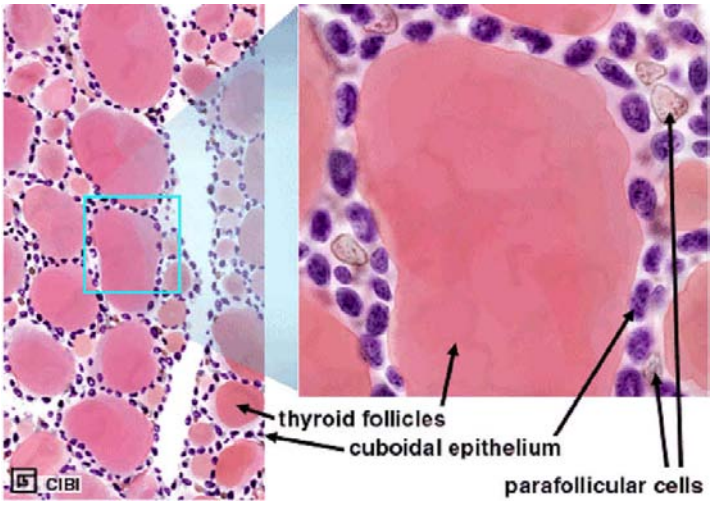
29.3. Recommended CAGs for the thymus

No CAGs for toxicity to the thymus are recommended.

30. Thyroid gland

30.1. Introduction

The thyroid gland is shaped like a butterfly and located on the trachea. The gland consists of thyroid follicles and small clusters of parafollicular cells in between the follicles. The thyroid follicles consist of thyroid follicular cells (cuboidal epithelium on Figure 30.1) surrounding a lumen filled with material called colloid.



From http://www.drharper.ca/new_page_12.htm

Figure 30.1. Histological section of a normal thyroid gland

The thyroid gland produces hormones that affect the function of almost every organ system in the body.

The parafollicular cells (also called C-cells) produce the hormone calcitonin which participates in the regulation of the calcium metabolism. Only few chemicals have been found to disturb the structure or function of the parafollicular cells.

The thyroid follicular cells produce iodine-containing hormones called iodothyronines. Thyroxine (T4) and triidothyronine (T3) are the most important iodothyronines. T3 and T4 play essential roles in the normal development of e.g. brain, testis, lungs, and heart in embryos and maturing animals. In adults, T3 and T4 regulate e.g. the energy metabolism and endocrine functions. Generally, chemicals that affect the thyroids change the structure or function of the follicular cells or change the concentration or peripheral action of T3 and T4.

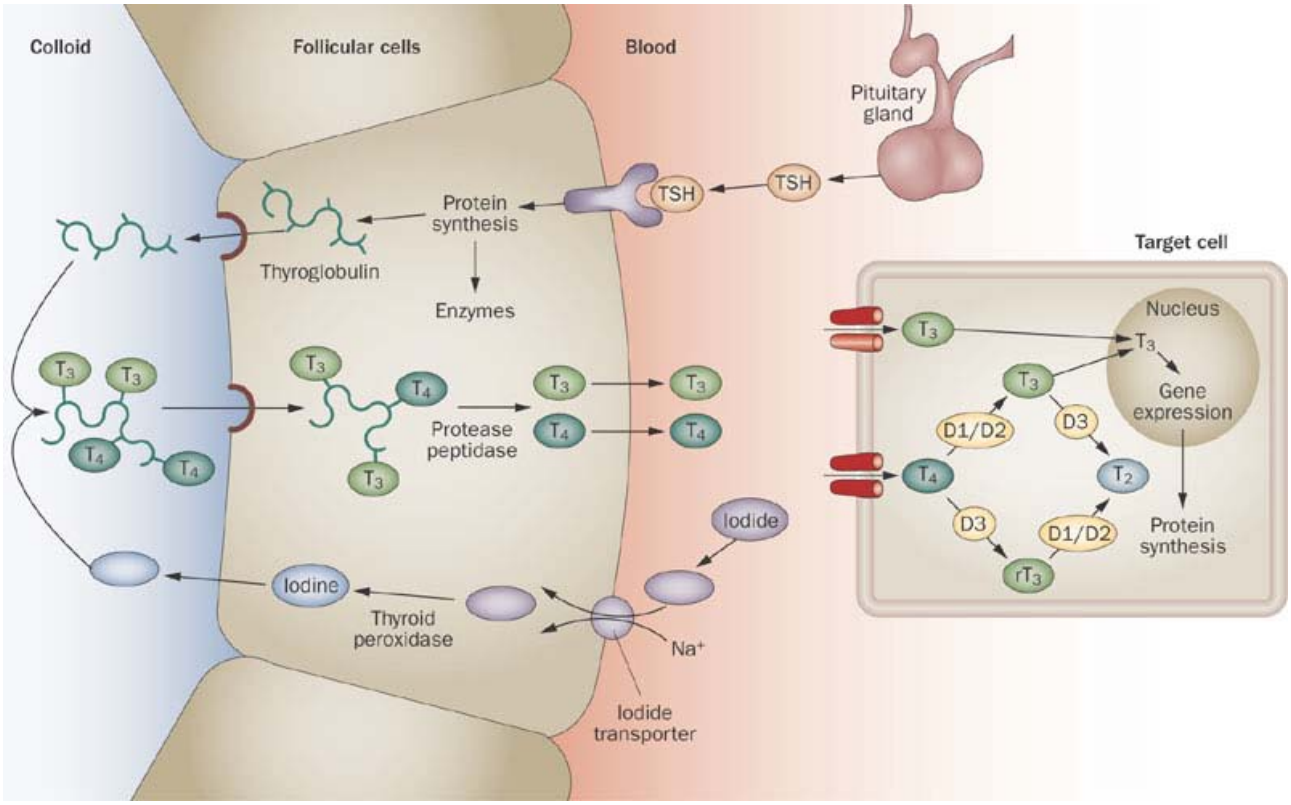
30.1.1. Regulation of circulating levels of T3 and T4

The function of the thyroid gland is primarily regulated via a feedback mechanism in which thyrotropin-releasing hormone (TRH) from the hypothalamus and thyroid-stimulating hormone (TSH) from the pituitary are released in response to decreased circulating levels of T3 and T4. TSH is essential for the thyroid gland to synthesize and secrete T3 and T4.

30.1.2. Synthesis and secretion of T3 and T4

The thyroid follicular cells, upon stimulation by TSH, synthesize thyroglobulin, which is released into the colloid-filled lumen inside the follicle (Figure 30.2). Iodide is actively transported into the thyroid follicular cells, where it is oxidized by thyroid peroxidase (TPO) into iodine and released into the colloid. Iodine binds to tyrosine residues within

thyroglobulin. T3 and T4 are synthesized within the thyroglobulin molecule via coupling of the resulting iodotyrosines. T3 and T4 are stored in the colloid within thyroglobulin. Following stimulation by TSH the thyroglobulin with T3 and T4 is reabsorbed into the follicular cells. Enzymes cleave the T3 and T4 molecules, and free T3 and T4 are released into the blood circulation.



From: Janna Cohen-Lehman, Peter Dahl, Sara Danzi & Irwin Klein (2010). Effects of amiodarone therapy on thyroid function. Nature Reviews Endocrinology 6, 34-41

Figure 30.2. Synthesis of T3 and T4

30.1.3. Transport of T3 and T4

In the blood circulation the major part of T3 and T4 is bound in reversible equilibrium to various serum proteins of which thyroxine-binding globulin (TBG) and transthyretin (TTR) are the most important. TBG is the major plasma T4-binding protein in humans whereas TTR is the major plasma T4-binding protein in rats. The effect of the binding proteins is to maintain the serum free T3 and T4 within narrow limits to ensure that the hormones are continuously and immediately available to the organs. In general, T3 is bound less tightly to the proteins than T4 resulting in a shorter plasma half-life of T3. T3 is therefore more susceptible to alterations in hormone production than T4.

The membranes of target cells for T3 and T4 contain transport proteins such as different organic anion-transporting polypeptides (OATPs). These transport proteins are responsible for moving T3, T4 and metabolites in and out of the cells.

30.1.4. Biological action of T3 and T4

The majority of biological actions of iodothyronines seem to be mediated through receptors for T3 in the target cells. T3 activates the energy metabolism when it binds to receptors on the inner mitochondrial membrane. T3 stimulates the synthesis of membrane proteins, enzymes and hormones when it binds to nuclear receptors.

30.1.5. Metabolism and excretion of T3 and T4

T4 is only produced in the thyroid gland. T3 is the physiologically active form of the iodothyronines. In humans about 80 % of circulating T3 comes from deiodination of T4. T4 is converted to T3 predominantly by type I iodothyronine deiodinase (D1). D1 is found largely in the kidney, liver and thyroid, while type II iodothyronine deiodinase (D2) is present primarily in skeletal muscle, the central nervous system and the pituitary. Type III iodothyronine deiodinase (D3), found in the brain, skin and placenta, inactivates T4 and T3, which leads to the formation of reverse T3 (rT3) and T2, respectively.

T3 and T4 are primarily excreted in bile after conjugation in the liver to glucuronic acid (maily T4) or sulphate (mainly T3). T3 and T4 are glucuronidated by different uridine diphosphate glucuronyl-tranferases (UDPGT) and sulphated by different sulphotransferases (SULT). Sulphate conjugation of T3 also enhances the conversion of T4 to T3 by D1.

UDPGT and SULT activities can be induced by a number of endogenous and exogenous compounds such as drugs and pesticides by various mechanisms i.e.. via nuclear receptors such as the pregnane x receptor (PXR), which also encodes for the induction of various cytochrome P450 enzymes, such as CYP3A4 in experimental animals and humans. Induction of these Phase II enzymes enhances the conjugation of T3 and T4.

Bacteria in the intestinal lumen hydrolyse biliary excreted conjugated T3 and T4. The hydrolysed hormones are partially reabsorbed.

30.1.6. Studies on effects on the thyroid in experimental animals

The active substances on the European market typically have been tested in a battery of standard toxicological tests. In the standard guidelines for testing of repeated dose toxicity including chronic toxicological studies in rodents, histopathological changes in the thyroid such as follicular cell hyperplasia or hypertrophy, different follicular cell tumours, and different colloid alterations will be revealed. However, the standard guidelines do not include as a mandatory requirement analysis of hormones such as T3, T4 and TSH as well as thyroid weight. However, in a number of the toxicological studies on active substances, thyroid hormones and weight have been measured.

30.2. Establishment of CAGs for toxicity to the thyroid gland

30.2.1. CAG level 1: Toxicity to the thyroid gland

The active substances identified as having an effect on the thyroid gland in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 30.1.

Table 30.1. CAG level 1: Toxicity to the thyroid gland

2,4-D	Flumioxazin	Propaquizafop
2,4-DB (metabolized to 2,4-D)	Fluopicolide	Propineb
Aclonifen	Fluoxastrobin	Propyzamide
Amidosulfuron	Flutolanil	Prothioconazole
Amitrole (aminotriazole)	Folpet	PTU (metabolite of probineb)
Beflubutamid	Formetanate	Pymetrozine
Benalaxyl	Fuberidazole	Pyrethrins
Benfluralin	Imazosulfuron	Pyrimethanil
Benthiavalicarb	Imidacloprid	Quizalofop-P-tefuryl
Boscalid	Ioxynil	Silthiopham
Bromoxynil	Isoxaflutole	Spinosad
Chlorpropham	Lenacil	Tepraloxydim
Clodinafop	Lufenuron	Tetraconazole
Clofentezine	Maleic hydrazide	Thiabendazole
Cyflufenamid	Mancozeb	Thiacloprid
Cyhalofop-butyl	Maneb	Thiamethoxam
Cyprodinil	MCPA	Thiophanate-methyl
Desmedipham	MCPB (metabolized to MCPA)	Thiram
Dinocap	Metiram	Tolylfluanid
EBIS (metabolite of mancozeb, maneb and metiram)	Metribuzin	Tribenuron (aka metometuron)
Etofenprox	Oxadiazon	Tritosulfuron
ETU (metabolite of mancozeb, maneb and metiram)	Pendimethalin	TTCA (metabolite of tolylfluanid)
Fenamidone	Pethoxamid	Ziram
Fipronil	Picloram	Zoxamide
Flufenacet (formerly fluthiamide)	Picolinafen	

30.2.2. CAG level 2: Phenomenological / specific effects on the thyroid gland

Effects were noted in the follicular cells as well as in the parafollicular cells.

Various types of effects on the follicular cells and altereations in thyroid hormones have been identified as a basis for establishing CAGs at level 2:

- Changes in serum T3 (and T4)
- Increased TSH
- Follicular cell hyperplasia/hypertrophy

- Follicular cell tumours.

Thyroid disrupting chemicals may damage the follicular cells and/or interfere with normal function generally resulting in decreased serum levels of T3 and/or T4. Hypothalamus and the pituitary gland detect the decreased level of thyroid hormones leading to increased secretion of TSH. Persistent elevation of TSH leads to thyroid follicular cell hypertrophy and hyperplasia and an enlarged thyroid gland. Experiments in rodent have shown that prolonged TSH-stimulation may lead to benign or even malignant follicular cell tumours. In some cases, chronic stimulation also results in pituitary hyperplasia and tumours (because of increased secretion of TRH).

Experiments in rodents have shown that chemicals that disturb the level of T3 and T4 and/or bind to thyroid hormone receptors may also cause developmental effects.

Epidemiologic studies have shown that adults with subclinical hypothyroidism (elevation in TSH with normal T4) have an increased risk of cardiovascular disease because of increased blood pressure and poorer blood lipid profiles. Miller et al. (2009) suggest that elevated risk of cardiovascular disease should be considered possible from exposure to thyroid disturbing chemicals.

The effects noted on the parafollicular cells were hyperplasia and tumours.

Based on the effects observed in the follicular and parafollicular cells, six distinct CAGs at level 2 are proposed. More information is given in Appendix AP.

30.2.2.1. CAG level 2a: Decreased T3 and/or T4

The active substances identified as decreasing T3 and/or T4 levels in serum are allocated to CAG level 2a and are listed in Table 30.2. It should be noted that in standard guidelines for toxicological studies analysis of hormones such as T3, T4 and TSH are not a mandatory requirement. It is therefore possible that more active substances with effects on the follicular cells might also have effects of T3/T4 levels.

Table 30.2. CAG level 2a: Decreased serum T3 and/or T4

2,4-D	Flufenacet (formerly fluthiamide)	Propyzamide
Aclonifen	Fluoxastrobin	Prothioconazole
Amitrole (aminotriazole)	Imidacloprid	PTU (metabolite of probineb)
Benthiavalicarb	Ioxynil	Pymetrozine
Boscalid	Isoxaflutole	Pyrethrins
Bromoxynil	Lenacil	Pyrimethanil
Chlorpropham	Lufenuron	Tetraconazole
Clofentezine	Mancozeb	Thiabendazole
Cyflufenamid	Maneb	Thiacloprid
Desmedipham	Metiram	Thiophanate-methyl
EBIS (metabolite of mancozeb, maneb and metiram)	Metribuzin	Tolylfluanid
Etofenprox	Oxadiazon	Tritosulfuron
ETU (metabolite of mancozeb,	Pendimethalin	Ziram

maneb and metiram)		
Fipronil	Propineb	

30.2.2.2. CAG level 2b: Increased TSH

The active substances identified as increasing TSH levels in serum are allocated to CAG level 2b and are listed in Table 30.3. It should be noted that in standard guidelines for toxicological studies analysis of hormones such as T3, T4 and TSH are not a mandatory requirement. It is therefore possible that more active substances with effects on the follicular cells might also have effects of TSH levels.

Table 30.3. CAG level 2b: Increased serum TSH

Aclonifen	Mancozeb	PTU (metabolite of probineb)
Amitrole (aminotriazole)	Maneb	Pymetrozine
Benthiavalicarb	Metiram	Pyrethrins
Boscalid	Metribuzin	Pyrimethanil
Clofentezine	Oxadiazon	Tetraconazole
Cyflufenamid	Pendimethalin	Thiabendazole
Etofenprox	Pethoxamid	Thiacloprid
ETU (metabolite of mancozeb, maneb and metiram)	Propineb	Thiophanate-methyl
Fipronil	Propyzamide	Tolylfluanid
Ioxynil	Prothioconazole	

30.2.2.3. CAG level 2c: Follicular cell hypertrophy / hyperplasia

Follicular cell hypertrophy is an increase in the size of the cells and hyperplasia is an increased number of the cells. These effects are often accompanied by increased organ weight.

Hypertrophy and hyperplasia frequently occur together and for the purpose of the CAG project these findings are allocated to a single CAG level 2, termed ‘CAG level 2a: Follicular cell hypertrophy / hyperplasia’.

The active substances identified as inducing one or more of the above-mentioned effects are allocated to CAG level 2c and are listed in Table 30.4.

Table 30.4. CAG level 2c: Follicular cell hypertrophy / hyperplasia

2,4-D	Flufenacet (formerly fluthiamide)	Picloram
2,4-DB (metabolized to 2,4-D)	Flumioxazin	Picolinafen
Aclonifen	Fluopicolide	Propaquizafop
Amidosulfuron	Fluoxastrobin	Propineb

Amitrole (aminotriazole)	Flutolanil	Propyzamide
Beflubutamid	Folpet	PTU (metabolite of probineb)
Benalaxyl	Formetanate	Pymetrozine
Benfluralin	Fuberidazole	Pyrethrins
Benthiavalicarb	Imazosulfuron	Pyrimethanil
Boscalid	Ioxynil	Quizalofop-P-tefuryl
Bromoxynil	Isoxaflutole	Silthiopham
Chlorpropham	Lenacil	Spinosad
Clodinafop	Lufenuron	Tepraloxydim
Clofentezine	Maleic hydrazide	Tetraconazole
Cyflufenamid	Mancozeb	Thiabendazole
Cyhalofop-butyl	Maneb	Thiacloprid
Cyprodinil	MCPA	Thiamethoxam
Desmedipham	MCPB (metabolized to MCPA)	Thiophanate-methyl
Dinocap	Metiram	Thiram
Etofenprox	Metribuzin	Tolylfluanid
ETU (metabolite of mancozeb, maneb and metiram)	Oxadiazon	Tritosulfuron
Fenamidone	Pendimethalin	Ziram
Fipronil	Pethoxamid	Zoxamide

30.2.2.4. CAG level 2d: Follicular cell neoplasms

The active substances identified as inducing follicular cell neoplasms are allocated to CAG level 2d and are listed in Table 30.5.

Table 30.5. CAG level 2d: Follicular cell neoplasms

2,4-D	Flufenacet (formerly fluthiamide)	Picloram
2,4-DB (metabolized to 2,4-D)	Flumioxazin	Picolinafen
Amitrole (aminotriazole)	ETU (metabolite of mancozeb, maneb and metiram)	Pethoxamid
Beflubutamid	Fipronil	Propyzamide
Benfluralin	Fuberidazole	Pyrethrins
Benthiavalicarb	Ioxynil	Silthiopham
Boscalid	Isoxaflutole	Thiabendazole
Cyflufenamid	Mancozeb	Thiacloprid
Etofenprox	Pendimethalin	Thiophanate-methyl

30.2.2.5. CAG level 2e: Parafollicular cell hyperplasia

The active substances identified as inducing parafollicular cell hyperplasia are allocated to CAG level 2e and are listed in Table 30.6.

Table 30.6. CAG level 2e: Parafollicular cell hyperplasia

2,4-D	Fenamidone	Thiram
2,4-DB (metabolized to 2,4-D)	Folpet	Ziram
Amitrole (aminotriazole)	Imidacloprid	
Desmedipham	Picloram	

30.2.2.6. CAG level 2f: Parafollicular cell neoplasms

Only one active substance was identified as inducing tumours in the parafollicular cells. Thiram induced an increased incidence of parafollicular cell adenomas in a 2-year rat dietary toxicity study. This substance is allocated to CAG level 2f and is listed in Table 30.7.

Table 30.7. CAG level 2f: Parafollicular cell neoplasms

Thiram		

30.2.2.7. Effects not considered relevant for CAGs at level 2

In addition to the above mentioned effects, the DARs have for some active substances also reported other hormonal and biochemical changes such as increased/decreased iodide uptake, decreased free T4 or T3, increased/decreased free T4 index, increased T3/T4 ratio, increased/decreased rT3, decreased T4 volume of distribution, increased/decreased T4 binding capacity, increased T4 binding to albumin, decreased T4 binding to TTR, increased/decreased plasma bound iodide, and decreased TBG.

The DARs have also reported other histopathological changes such as increased/decreased amount of colloids, small/large follicles, different shapes of the follicular cells, increased vascularization, increased vacuolation, follicular cysts, follicular cell pigmentation, follicular atrophy, necrosis of follicular cells, and ectopic thyroid tissue surrounding the aortic arch.

CAGs were not created for the above mentioned effects as many of the histopathological changes are a consequence of hyperactivity of the thyroid gland which is already covered by the CAG level 2c: Follicular cell hypertrophy / hyperplasia. Anyhow the number of active substances causing e.g. follicular cell pigmentation or necrosis of follicular cells is limited.

The additional hormonal and biochemical changes have only been studied for a limited number of active substances. They may help in making hypothesis for the mechanisms behind the thyroid disturbing effect but is considered of limited relevance on their own.

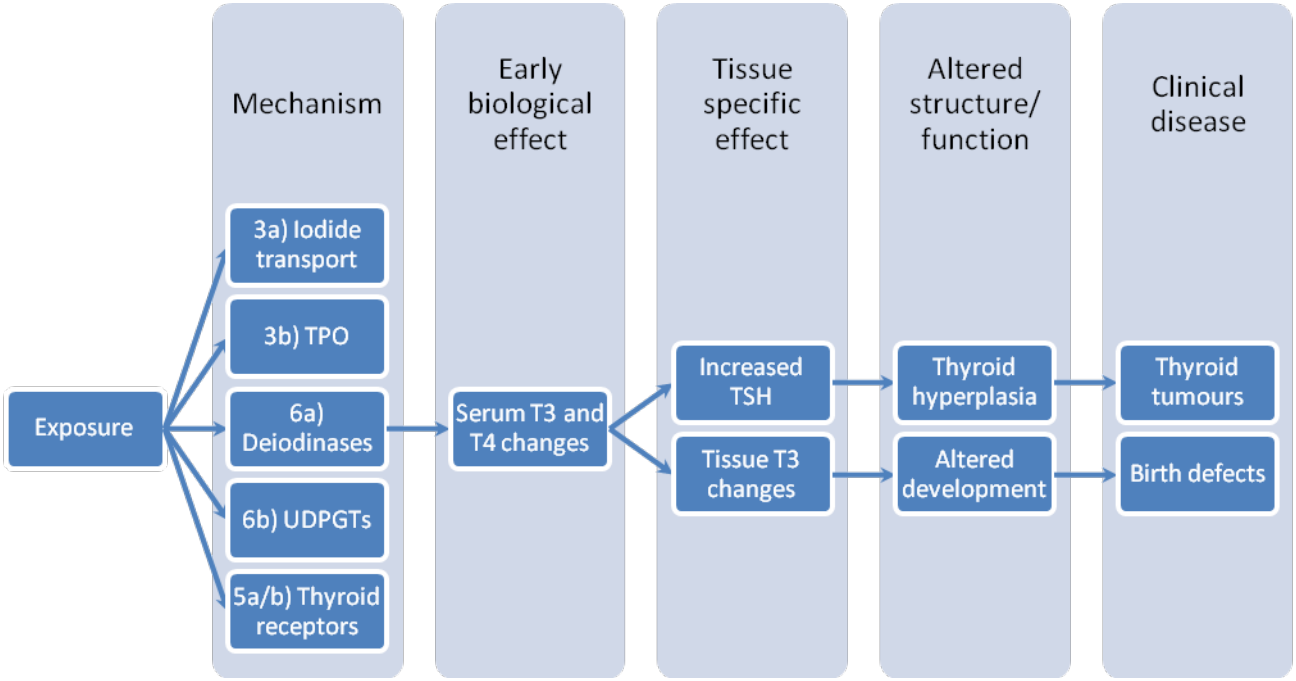
For three active substances, an increased incidence of thyroglossal duct cysts or ultimobranchial cysts was reported. These cysts are found in remnants of embryological anatomical structures. Thyroglossal duct cysts may form in a thyroglossal duct that fails to atrophy. The thyroglossal duct forms an open connection between the initial area of

development of the thyroid gland and its final position. Ultimobranchial cysts are remnants of the ultimobranchial body, responsible for the distribution of calcitonin secreting C-cells in the thyroid. These cysts are not considered to be treatment-related, because the persistence of embryonic structures is a congenital lesion and could not have been influenced by the test compound.

30.2.3. CAG level 3: Mode of action

For some of the phenomenological / specific effects on the thyroid described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

A combined mode of action model for the effects of thyroid follicular cell disturbing chemicals is outlined in Figure 30.3. The specific mechanisms are described in more detail under CAG level 4.



Modified from Crofton (2008) and Miller et al. (2009)

Figure 30.3. A combined mode of action model for the effects of thyroid follicular cell disturbing chemicals on cancer and developmental effects

As already described under CAG level 2, the different effects on the thyroid follicular cells or on the thyroid hormone levels are often not independent effects but rather consequences of each other. The combined mode of action model illustrates that many different mechanisms of action may lead to serum T3 and T4 changes. Subsequently TSH is increased to compensate

for the decreased T3 and/or T4. Persistent elevation of TSH may lead to histopathological changes such as thyroid follicular cell hypertrophy and even thyroid follicular cell tumours (at least in rodents).

The combined mode of action model also illustrates that chemicals that disturb the levels of T3 and T4 and/or bind to the thyroid hormone receptors may also cause developmental effects.

In order to establish a CAG at level 3 (mode of action) it was decided to group the active substances or metabolites that were found to fit into this combined mode of action model based on the criteria that at least two of the following effects have been observed for the substance:

- Effect on one or more of the above mentioned mechanisms
- Serum T3 and/or T4 changes
- Increased TSH
- Follicular cell hypertrophy/ hyperplasia and/or increased thyroid weight
- Follicular cell tumours

30.2.3.1. CAG level 3: Follicular cell toxicity (combined mode of action model)

The active substances fulfilling the criteria for fitting to the combined mode of action model are allocated to CAG level 3 and are listed in Table 30.8.

Table 30.8. CAG level 3: Follicular cell toxicity (combined mode of action model)

2,4-D	Fipronil	Propineb
Aclonifen	Flufenacet (formerly fluthiamide)	Propyzamide
Amitrole (aminotriazole)	Fluoxastrobin	Prothioconazole
Beflubutamid	Fuberidazole	PTU (metabolite of probineb)
Benfluralin	Ioxynil	Pymetrozine
Benthiavalicarb	Isoxaflutole	Pyrethrins
Boscalid	Lenacil	Pyrimethanil
Bromoxynil	Lufenuron	Silthiopham
Chlorpropham	Mancozeb	Tetraconazole
Clodinafop	Maneb	Thiabendazole
Clofentezine	Metiram	Thiacloprid
Cyflufenamid	Metribuzin	Thiophanate-methyl
Desmedipham	Oxadiazon	Tolylfluanid
Etofenprox	Pendimethalin	Tritosulfuron
ETU (metabolite of mancozeb, maneb and metiram)	Pethoxamid	Ziram

30.2.4. CAG level 4: Mechanism of action

Thyroid disrupting chemicals may damage the follicular cells and/or interfere with normal function at almost all steps of the regulation, synthesis, secretion, transport, biological action, metabolism and excretion of T3 and T4, generally resulting in decreased plasma levels of T3 and/or T4.

The known mechanisms that may lead to thyroid toxicity are listed in the following. Not all of the mentioned mechanisms are relevant for the active substances of this project.

1. Damage of the follicular cells

1a) Tumours due to mutagenic properties of the chemical

1b) Inflammation and/or degeneration of the thyroid gland

2. Interference with regulation

2a) Induced inability of the hypothalamus and/or pituitary gland to produce TRH and TSH, respectively

2b) Decreased response of TSH-producing cells to TRH

3. Interference with synthesis and secretion

3a) Inhibition of the active iodide transport into the thyroid follicular cells

3b) Inhibition of thyroid peroxidise (TPO)

3c) Inhibition of T3 and T4 secretion

3d) Disruption of T3 and T4 synthesis because of accumulation of the chemical in the thyroid gland

4. Interference with transport of the thyroid hormones

4a) Competitive displacement of T4 from its binding site on TTR. No similar effect has at present been shown with TBG

4b) Up-regulation of cellular transport proteins such as OATPs

5. Interference with the biological action

5a) Agonist to the biological actions of T3

5b) Antagonist to the biological actions of T3

The underlying mechanisms for interference with the biological action may be binding to the thyroid receptor or interference with its expression. A thyroid disrupting chemical may also affect one or more of the processes occurring between T3 receptor binding and transcription of the target genes

6. Interference with metabolism and excretion

6a) Inhibition of the different iodothyronine deiodinases

6b) Induction of UDPGT and/or SULT

6c) Inhibition of SULT

6d) Reduced re-absorption of T3 and T4 from the intestine

Table 30.9 outlines how the levels of circulating thyroid hormones and TSH in theory may change depending on the underlying mechanism of action.

Table 30.9. Circulating thyroid hormone and TSH levels after different mechanisms of action

	Circulating thyroid hormone and TSH levels	Other effects
Damage of the follicular cells		
1a) Tumours due to mutagenic properties of the chemical	?	Positive in mutagenic experiments Neoplasi in the thyroid
1b) Inflammation and/or degeneration of the thyroid gland	Decreased T3 and T4 Increased TSH (and TRH)	Inflammation/degeneration in the thyroid
Interference with regulation	Decreased T3 and T4 Decreased TSH	
2a) Induced inability of the hypothalamus and/or pituitary gland to produce TRH and TSH, respectively	(Decreased or increased TRH depending on the function of hypothalamus)	
2b) Decreased response of TSH-producing cells to TRH	(Increased TRH)	
Interference with synthesis and secretion	Decreased T3 and T4 Increased TSH (and TRH)	
3a) Inhibition of the active iodide transport into the thyroid follicular cells		
3b) Inhibition of TPO		
3c) Inhibition of T3 and T4 secretion		
3d) Disruption of T3 and T4 synthesis because of accumulation of the chemical in the thyroid gland		
Interference with transport		
4a) Competitive	?	

displacement of T4 from its binding site on TTR.		
4b) Upregulation of cellular transport proteins such as OATPs	Decreased T3 and T4 Increased TSH (and TRH)	
Interference with biological action	Not necessarily affected	
5a) Agonist to the biological actions of T3		
5b) Antagonist to the biological actions of T3		
Interference with metabolism and excretion		
6a) Inhibition of the different iodothyronine deiodinases	D1/D2: Decreased T3 Increased T4 Increased rT3 Increased TSH	
6b) Induction of UDPGT and/or SULT	UDPGTs for both T3 and T4: Decreased T3 and T4 Increased TSH (and TRH) Only for T4: Lesser effect on T3 and TSH SULT: ?	
6c) Inhibition of SULT	?	
6d) Reduced reabsorption of T3 and T4 from the intestine	?	

However, in practice it is not possible to distinguish between the different mechanisms only based on the level of thyroid hormones because:

- Chemicals may affect several mechanisms at the same time.
- Most of the mechanisms result in a decreased level of T3 and T4 and an increased level of TSH.
- The level of circulating thyroid hormones and TSH may change during experiments because of the ability of the body to compensate for the thyroid disturbing effect.

McClain et al. (1988, 1989) e.g. has shown that phenobarbital induces hepatic UDPGT resulting in increased elimination of T4. As expected plasma T3 and T4 was markedly decreased and TSH was increased at week 1 and 4. However, by week 8 T3 levels returned to near normal and the TSH level declined.

For a specific active substance where many experiments have been performed it is therefore common to see changes in especially the level of T3 between experiments.

Follicular cell hyperplasia or hypertrophy, an enlarged thyroid gland and follicular cell tumours are a consequence of persistent elevation of TSH irrespective of the underlying

mechanism. Therefore, most of the histopathological changes of the thyroid cannot be used to elucidate anything about the underlying mechanism of action.

Mechanistic studies are necessary to distinguish between most of the mechanisms. Only for about half of the active substances with effects on the thyroid, mechanistic studies have been performed to try to elucidate the mechanism behind the thyroid effect. Therefore, there is a lack of relevant mechanistic data for many of the active substances.

In the following CAGs at level 4 are established for active substances based on the current knowledge about their mechanisms of action. For many of the active substances with effect on the thyroid comments are made in the DAR that the effect was likely caused by liver enzyme induction. We have however not included such active substances in a CAG unless a mechanistic study has shown induction of UDPGT or SULT.

30.2.4.1. CAG level 4a: Inflammation and/or degeneration

The active substances allocated to CAG level 4a are listed in Table 30.10.

Table 30.10. CAG level 4a: Thyroid follicular cell toxicity related to inflammation and/or degeneration of the thyroid gland

Pymetrozine	Spinosad	Tribenuron

30.2.4.2. CAG level 4b: Inhibition of active iodide transport

The active substances allocated to CAG level 4b are listed in Table 30.11.

Table 30.11. CAG level 4b: Thyroid follicular cell toxicity related to inhibition of the active iodide transport into the follicular cells

Amitrole	Mancozeb	Metiram
ETU (metabolite of mancozeb, maneb, metiram)	Maneb	

30.2.4.3. CAG level 4c: Inhibition of TPO

The active substances allocated to CAG level 4c are listed in Table 30.12.

Table 30.12. CAG level 4c: Thyroid follicular cell toxicity related to inhibition of TPO

Amitrole	Metiram	Thiophanate-methyl

ETU (metabolite of mancozeb, maneb, metiram)	Propineb	TTCA (metabolite of tolylfluanid)
Mancozeb	PTU (metabolite of propineb)	Ziram
Maneb		

30.2.4.4. CAG level 4d: Competitive displacement of T4 from its binding to TTR

The active substances allocated to CAG level 4d are listed in Table 30.13.

Table 30.13. CAG level 4d: Follicular cell toxicity related to competitive displacement of T4 from its binding to TTR

Ioxynil		

30.2.4.5. CAG level 4e: Antagonism to T3 action

The active substances allocated to CAG level 4e are listed in Table 30.14.

Table 30.14. CAG level 4e: Follicular cell toxicity related to antagonism to T3 action

Etofenprox	PTU (metabolite of propineb)	

30.2.4.6. CAG level 4f: Inhibition of iodothyronine deiodinases

The active substances allocated to CAG level 4f are listed in Table 30.15.

Table 30.15. CAG level 4f: Follicular cell toxicity related to inhibition of iodothyronine deiodinases

ETU (metabolite of mancozeb, maneb, metiram)	Propineb	TTCA (metabolite of tolylfluanid)
Flufenacet	PTU (metabolite of propineb)	

30.2.4.7. CAG level 4g: Induction of UDPGT and/or SULT

The active substances allocated to CAG level 4g are listed in Table 30.16.

Table 30.16. CAG level 4g: Follicular cell toxicity related to induction of UDPGT and/or SULT

Benthiavalicarb	Flufenacet	Prothioconazole
Boscalid	Fluoxastrobin	Pymetrozine
Chlorpropham	Isoxaflutole	Pyrethrins
Clodinafob	Metribuzin	Pyrimethanil
Clofentezin	Pendimethalin	Thiabendazole
Cyflufenamid	Pethoxamid	Thiacloprid
Etofenprox	Propyzamide	Thiophanate-methyl
Fipronil		

30.3. Discussion of CAGs for the thyroid gland

Sixty active substances were identified to have effects on the thyroid and were allocated to CAG level 1. Six distinct CAGs at level 2 have been proposed. Information on modes/mechanisms of action is available for some of the active substances. The information is summarised in Appendices AQ, AR and AS.

The mechanism of action is known for about half of the active substances with effects on the thyroid follicular cells. However, if an active substance is likely to fit into the combined mode of action model established, the detailed mechanism of action might not be essential for cumulative risk assessment.

Generally dose addition models are used to predict the response to mixtures of chemicals that act through the same mechanism. Response addition models are used to calculate the response to mixtures containing chemicals acting through different mechanisms but with a common response e.g. decreased circulating T4 concentrations. Integrated addition models are used to predict the response to a mixture containing chemicals with similar and dissimilar mechanisms that affect a common downstream end point such as serum T4.

Crofton et al. (2005) investigated a mixture of 18 thyroid disturbing chemicals. All 18 chemicals tested one by one decreased the level of circulating T4. One mechanism by which these chemicals alter the T4 concentration is thought to be via upregulation of the hepatic enzyme UDPGT. However, different mechanisms may lie behind this change. Some of the chemicals tested were known to bind to the Aryl-hydrocarbon-receptor. Others were known to bind to other receptors such as the PXR. Crofton et al. showed that the mixture caused decreases in the T4 concentration even though the individual chemical concentrations in the mixture were below effective doses. Dose addition predicted the effect (decreased T4) of the mixture with a fair degree of accuracy whereas effect addition underestimated the effect.

Along the same lines, Flippin et al. (2009) investigated a mixture of 21 thyroid disturbing chemicals of which 18 stimulate T4 clearance in the liver and 3 inhibit thyroid hormone synthesis. The results of dose addition and integrated addition models were similar, and both provided better predictions than the effect addition model.

These two studies suggest that it may be possible to predict a decreased level of T4 in a mixture of chemicals with a fair degree of accuracy using dose addition models without knowing the detailed mechanism behind the decreased T4 level.

It is therefore recommended to consider the CAG level 3: Mode of action for effects on follicular cells" for cumulative risk assessment. It should be noted that hormone levels have been studied for most but not all of the active substances listed under CAG level 3.

In toxicological test guideline studies it is not a requirement to measure the level of T3, T4 and TSH. Therefore, active substances may have influenced the level of the thyroid hormones without being listed under CAG level 2a (Decreased T3 and/or T4) and CAG level 2b (Increased TSH) simply because the hormone levels were not measured.

As follicular cell tumours are a consequence of prolonged increased levels of TSH, they are often found only at the highest dose levels and only with a slightly increased incidence compared to the control group. Therefore, it is not always clear from the DARs or peer review reports (if available) whether the experts consider the tumours treatment related.

Humans, monkeys, guinea pigs, and chickens seem to be less sensitive than rats, mice and dogs to thyroid disturbances even though the basic hypothalamic-pituitary-thyroid axis functions in a similar way in humans and animals. Rats are more sensitive than mice which are more sensitive than dogs. Particularly male rats are considered highly sensitive for thyroid disturbances due to higher circulating levels of TSH. The following are some of the species differences that may explain sensitivity differences:

- Differences in the concentration of chemicals needed to inhibit TPO. Monkeys are e.g. less sensitive than rats.
- Differences in the transport of T3 and T4. In humans and monkeys, for instance, T4 is mainly bound to TBG whereas in rodents T4 is mainly bound to TTR. TBG has a binding affinity for T4 approximately 1000 times higher than TTR. As a result, the plasma half-life of T4 is much shorter in rodents than in humans. The consequence of a shorter plasma half life of T4 is a greater susceptibility in rodents for alterations in the T4 production or break down. In addition, competitive displacement of T4 from its binding site has so far only been shown for TTR and not for TBG.
- Differences in the activity of deiodinases. The activity of D1 is lower in human than in rat liver.
- Differences in the importance of glucuronidation of T3 and T4 for excretion. Glucuronidation is less important in humans than in rats.
- Differences in the efficiency of the enterohepatic cycle. The efficiency may be greater in humans than in rats.

Because of these differences it is generally accepted that non-genotoxic chemicals, which cause thyroid tumours in rats and mice because of changes in the thyroid and pituitary hormone levels, will not cause thyroid tumours in humans. However, even if the results of rodent experiments may not reflect an accurate measure of the degree of thyroid disturbances

in humans, they do indicate whether a chemical has the potential to change the level of T3 and T4 in humans exposed to high enough doses.

Therefore, the relevance for humans to a great extent is a question of dose, i.e. whether the exposure to a thyroid disturbing chemical is sufficient to cause effects. Even small and transient changes in the T3 and T4 levels in humans may affect the developing nervous system in the unborn children. To make it even more complicated, the duration and timing of T3 and T4 insufficiency are also important and may vary by species.

30.4. Recommended CAGs for the thyroid gland

The following CAG at level 3 is recommended for CRA for effects on the thyroid gland:

- CAG level 3: Follicular cell toxicity (combined mode of action model), see Table 30.8.

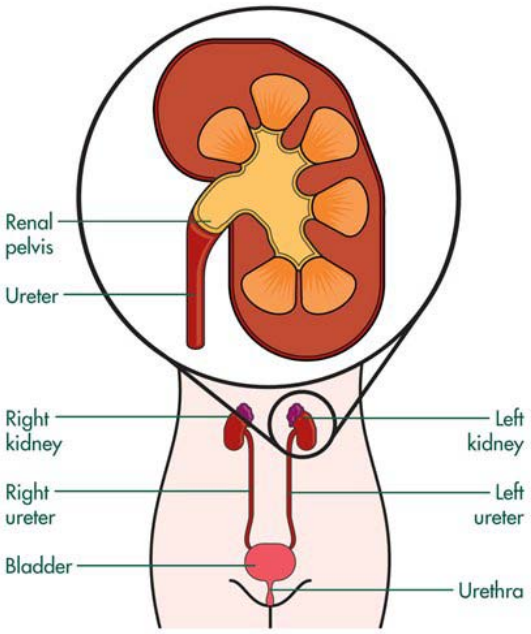
31. Urinary bladder

31.1. Introduction

The urine formed by the nephrons in the kidney flow from the distal tubules and collecting ducts through the renal papilla, and into the calyces, and is collected in the renal pelvis. From the renal pelvis, urine is funnelled into the ureters. Peristaltic activity of the ureters propels urine into the bladder. Peristalsis is controlled by the nervous system and by the urine volume.

The bladder is a bag composed of smooth muscle fibres and transitional epithelium. Its function is to store urine until elimination. As the bladder fills with urine, it distends and the layers of transitional epithelium slide past each other and become thinner as the volume of the bladder increases. The urethra extends from the inferior side of the bladder to the outside of the body. Two muscles called sphincters control excretion of urine from the bladder through the urethra. One of the sphincters is under voluntary control. The other sphincter and the bladder are under control of the nervous system via impulses from mechanoreceptors in the bladder. (McCance and Huether 1998).

The urinary passages, the renal pelvis, the ureters, the bladder and the urethra are histological similar to each other. They have a mucosal layer lined with transitional epithelium. Underlying the mucosal layer are muscle layers covered by a serous membrane or connective tissue. (Verlander 1998). Therefore the pathogenesis for effects in the urinary bladder may be the same as for effects in the renal pelvis, ureters and urethra (Cohen 1998). Effects on the renal pelvis and ureters have been included in the chapter about the kidney



From http://www.macmillan.org.uk/Cancerinformation/Cancertypes/Kidney/Aboutkidneycancer/Ureterrenalpelvis.aspx

Figure 31.1. Anatomy of the urinary passages

31.2. Establishment of CAGs for toxicity to the urinary bladder

31.2.1. CAG level 1: Toxicity to the urinary bladder

The active substances identified as having an effect on the urinary bladder in the toxicological studies included in the DARs are allocated to CAG level 1 and are listed in Table 31.1.

Table 31.1. CAG level 1: Toxicity to the urinary bladder

2-Phenylphenol	Flusilazole	Pyrimethanil
Aclonifen	Fosetyl	Quinoclamine
Azimsulfuron	Glyphosate	Spiroxamine
Chloridazon (metabolite)	Iprodione	Sulfosulfuron
Chlorothalonil	Lufenuron	Tepraloxydim
Clodinafop	Maleic hydrazide	Thiabendazole
Cymoxanil	Metazachlor	Triasulfuron
Cyromazine (metabolite)	Oxasulfuron	Tritosulfuron
Diuron	Phenmedipham	Ziram
Fenpropidin	Prothioconazole	
Fluoxastrobin	Pyraflufen-ethyl	

31.2.2. CAG level 2: Phenomenological / specific effects on the urinary bladder

Various types of effects on the urinary bladder were identified as a basis for establishing CAGs at level 2. Based on these effects, four distinct CAGs at level 2 are proposed. More information is given in Appendix AT.

31.2.2.1. CAG level 2a: Hypertrophy / hyperplasia

Hypertrophy is an increased size of cells. Hyperplasia is an increased number of cells. It is mainly hyperplasia that has been noted in the urinary bladder. Hyperplasia of the urinary bladder may be a response to epithelial irritation, which in turn can be induced by urinary crystals, calculi or toxic chemicals (Fukushima and Murai 1999).

For the purpose of the CAG project, hypertrophy and hyperplasia are interpreted as representing the same type of effect in the urinary bladder and therefore, are allocated to a single CAG level 2, termed 'CAG level 2a: Hypertrophy / hyperplasia'. See Annex AT for a list of all terms in the DARs interpreted to represent hypertrophy and/or hyperplasia of the urinary bladder.

The active substances identified as inducing hypertrophy and/or hyperplasia in the urinary bladder are allocated to CAG level 2a and are listed in Table 31.2.

Table 31.2. CAG level 2a: Hypertrophy / hyperplasia in the urinary bladder

2-Phenylphenol	Fluoxastrobin	Pyrimethanil
Aclonifen	Flusilazole	Quinoclamine
Azimsulfuron	Fosetyl	Spiroxamine
Chloridazon (metabolite)	Glyphosate	Sulfosulfuron
Chlorothalonil	Iprodione	Tepraloxydim
Clodinafop	Maleic hydrazide	Thiabendazole
Cymoxanil	Metazachlor	Triasulfuron
Cyromazine (metabolite)	Oxasulfuron	Tritosulfuron
Diuron	Prothioconazole	Ziram
Fenpropidin	Pyraflufen-ethyl	

31.2.2.2. CAG level 2b: Cell degeneration / cell death

Necrosis and ulceration have predominantly been reported in the DARs for the active substances allocated to CAG level 2b. See Annex AT for a list of all terms in the DARs interpreted to represent cell degeneration / cell death of the urinary bladder.

The active substance identified as inducing cell degeneration / cell death are allocated to CAG level 2b and are listed in Table 31.3.

Table 31.3. CAG level 2b: Cell degeneration / cell death in the urinary bladder

2-Phenylphenol	Diuron	Sulfosulfuron
Cyromazine (metabolite)	Flusilazole	Tepraloxydim

Calculi may cause erosion and ulceration of the bladder epithelium (Cohen 1998). For four of the active substances (2-phenylphenol, cyromazine (metabolite), flusilazole, and sulfosulfuron) that induce cell death, calculi have also been noted. However, for 2-phenylphenol calculi does not seem to be considered part of the mode or mechanism of action for urinary bladder toxicity (see section 31.2.4.2). For 2-phenylphenol and diuron oxidative stress in the bladder epithelium has been measured and may form part of the mode of action for cell death.

31.2.2.3. CAG level 2c: Inflammation

Inflammation of the urinary bladder is commonly a response to epithelial irritation, which in turn can be induced by urinary crystals, calculi or toxic chemicals. It may be accompanied by hyperplasia. (Hard et al. 1999). See Annex AT for a list of all terms in the DARs interpreted to represent inflammation of the urinary bladder.

The active substances identified as inducing inflammation is allocated to CAG level 2c and are listed in Table 31.4..

Table 31.4. CAG level 2c: Inflammation in the urinary bladder

Aclonifen	Fluoxastrobin	Prothioconazole
Azimsulfuron	Flusilazole	Pyrimethanil
Clodinafop	Fosetyl	Quinoclamine
Cymoxanil	Iprodione	Sulfosulfuron
Cyromazine (metabolite)	Lufenuron	Tritosulfuron
Diuron	Oxasulfuron	
Fenpropidin	Phenmedipham	

31.2.2.4. CAG level 2d: Neoplasms

Errors do occur during DNA replication. Therefore with an increasing number of cells replications (hyperplasia) an adequate number of mistakes may be generated to lead to an increased incidence of neoplasms in life-time studies (Cohen 1998). As all of the active substances which cause neoplasms of the urinary bladder are considered non-genotoxic by the DARs and cause hyperplasia of the bladder epithelium, it is likely that the hyperplasia is part of the mode of action for the neoplasms. See Annex AT for a list of all terms in the DARs interpreted to represent neoplasms of the urinary bladder.

The active substances identified as inducing neoplasms are allocated to CAG level 2d and are listed in Table 31.5.

Table 31.5. CAG level 2d: Neoplasms in the urinary bladder

2-Phenylphenol	Diuron	Metazachlor
Aclonifen	Flusilazole	Quinoclamine
Cyromazine (metabolite)	Fosetyl	Sulfosulfuron

31.2.2.5. Effects not considered relevant for CAGs at level 2

Dilation:

Very large calculi can completely fill and distend the bladder lumen (Hard et al. 1999). Calculi have been found in the urinary bladder and/or in the kidney for all three active substances where dilation was noted. For these substances dilation of the urinary bladder is considered secondary to the effect already covered by the CAG level 3a1 for calculi, see below.

Oedema:

Oedema may accompany inflammation (Hard et al. 1999). Oedema in the urinary bladder has been found for two active substances together with inflammation. For these substances oedema in the urinary bladder is considered secondary to the effects already covered in the CAG level 2c for inflammation, see below.

Haemorrhage:

Haemorrhage in the urinary bladder may occur in conjunction with necrosis and inflammation (Hard et al. 1999, Cohen 1998). Haemorrhage in the urinary bladder has been found for three active substances together with necrosis and/or inflammation. For these substances haemorrhage in the urinary bladder is considered secondary to the effects already covered in the CAG level 2b for cell degeneration and/or cell death or in the CAG level 2c for inflammation.

Fibrosis:

Fibrosis is the formation of excess connective tissue in a reaction to toxicity of the urinary bladder. Fibrosis in the urinary bladder has been found for two substances together with inflammation and hyperplasia. For these substances fibrosis is considered secondary to the effects already covered especially in the CAG level 2c for inflammation.

Hyaline droplets:

Hyaline droplets are lysosomes containing protein. Hyaline droplets in the urinary bladder have only been reported for one substance.

Arteritis:

Arteritis is inflammation of the walls of arteries. Arteritis in the urinary bladder has only been reported for one substance and is not considered an effect specific for the urinary bladder.

Vacuolation:

Vacuolation of the transitional epithelium of the urinary bladder is a non-specific lesion as a response to cell injury. Eosinophilic inclusions often lie within the vacuoles and may represent cytoplasmic degradative products. Vacuolation may also be artefactual resulting from autolysis. (Hard et al 1999). Vacuolation has only been reported for two active substances where other kinds of cell injury such as necrosis and/or inflammation also have been noted.

Squamous metaplasia:

The epithelial cells are transformed into squamous epithelial cells typically as a response to irritation. Squamous metaplasia of the urinary epithelium has only been noted for two active substances where hyperplasia and/or neoplasms were noted in other studies. Although squamous metaplasia is not the same as hyperplasia or neoplasms, the squamous metaplasia is considered covered by the CAG level 2a for hypertrophy/hyperplasia and/or the CAG level 2d for neoplasms.

Dysplasia:

Dysplasia is delayed cell maturation and differentiation. Dysplasia is often indicative of an early neoplastic process. Dysplasia of the urinary epithelium has only been noted for one active substance where hyperplasia was noted in other studies. Although hyperplasia and dysplasia is not the same, the dysplasia is considered covered by the CAG level 2a for hypertrophy/hyperplasia.

31.2.3. CAG level 3: Mode of action

For some of the phenomenological / specific effects on the urinary bladder described under CAG level 2, a mode of action has been proposed. For the remaining substances, no information regarding mode of action has been found and consequently, these substances cannot be allocated to a CAG level 3.

31.2.3.1. CAG level 3a1: Calculi

Calculi are stones. Calculi may cause erosion and ulceration of the bladder epithelium with hemorrhage, inflammation and regeneration. Severe damage to the epithelium may lead to hyperplasia and ultimately neoplasms. Formation of calculi requires the concentration of the critical substances (e.g. active substance, metabolite, calcium) in the urine to be sufficiently high to lead to precipitate formation and ultimately to calculi. This can be influenced by specific chemical and physical condition of the urine (e.g. pH, volume). Urinary bladder calculi irrespective of composition may cause irritation and cell proliferation also in humans. The risk in humans may not be as great as that in rodents because the calculi are usually

voided spontaneously or removed by surgical procedures. As rodents are positioned horizontally they are less likely to eliminate the calculi spontaneously. (IARC 1999, Cohen 1998). See Annex AT for a list of all terms in the DARs interpreted to represent calculi.

The active substances identified as inducing calculi are allocated to CAG level 3a1 and are listed in Table 31.6.

Table 31.6. CAG level 3a1: Calculi

2-Phenylphenol	Flusilazole	Pyrimethanil
Chloridazon (metabolite) – in kidney	Fosetyl	Sulfosulfuron
Cyromazine (metabolite)	Maleic hydrazide	Thiabendazole
Fluoxastrobin	Oxasulfuron	Triasulfuron

During the course of an experiment calculi frequently are passed by the animals without being detected by the investigators because they are too small for gross visual detection or they have dissolved in the urine (Cohen 1998). Therefore, more active substances than those listed may cause urinary bladder toxicity by a calculi mode of action.

Chloridazon (metabolite) has been included in CAG level 3a1 even though calculi have not been detected in the bladder but only in the kidney as the substance clearly has the potential to produce calculi.

All of the twelve active substances, which induce calculi, also induce hyperplasia of the bladder. However it is only 2-phenylphenol, cyromazine (metabolite), flusilazole, and sulfosulfuron, which induce cell death in the bladder. And it is only cyromazine (metabolite), fluoxastrobin, flusilazole, fosetyl, oxasulfuron, pyrimethanil, and sulfosulfuron, which induce inflammation in the bladder.

Even though studies with 2-phenylphenol have shown calculi in the bladder, calculi does not seem to be considered part of the mode of action for 2-phenylphenol by the DAR. For cyromazine (metabolite), fluoxastrobin and fosetyl, calculi have been discussed as part of the mode/mechanism of action for these substances (see the section on mechanism of action). For the rest of the active substances, which induce calculi, no mode/mechanism of action for the bladder toxicity was discussed in the DARs.

31.2.3.2. CAG level 3a2: Crystals

Like calculi, crystals in the urine may lead to erosion of the bladder that may result in regenerative hyperplasia and ultimately neoplasms usually without inflammation. (Cohen 1998). See Annex AT for a list of all terms in the DARs interpreted to represent crystals.

The active substances identified as inducing crystals are allocated to CAG level 3a2 and are listed in Table 31.7

Table 31.7. CAG level 3a2: Crystals

Aclonifen	Cyromazine (metabolite)	Prothioconazole
Azimsulfuron	Fluoxastrobin	Sulfosulfuron
Chloridazon (metabolite)	Iprodione	

All of the eight active substances, which induce crystals, also induce hyperplasia of the bladder. Half of the substances also induce calculi (chloridazon (metabolite), cyromazine (metabolite), fluoxastrobin, and sulfosulfuron). The other half of the substances induce inflammation but not cell death in the bladder (aclonifen, azimsulfuron, iprodione, and prothioconazole).

31.2.3.3. CAG level 3e1: Oxidative stress

Oxidative stress occurs in cells when the concentration of reactive oxygen species (ROS) generated exceeds the antioxidant capability. ROS can be produced in normal cellular metabolism or by inflammatory cells. Reactive intermediate metabolites produced in the metabolism of xenobiotics may enhance the formation of ROS. Antioxidants such as vitamin C, vitamin E, glutathione (GSH), superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase normally inactivate ROS. With excessive formation of reactive intermediate metabolites and ROS the antioxidant capacity may be overloaded. The result is oxidative stress, which may result in damage to DNA, lipids, and proteins in the cell. Unrepaired DNA damage may lead to new mutations and potentially tumours. Oxidative injury may also produce cell death, which may lead to regenerative hyperplasia and ultimately tumours. (Klaunig et al. 1998).

Active substances, which induce oxidative stress measured as a decreased level of the antioxidant GSH are included in this CAG.

The active substances identified as inducing oxidative stress are allocated to CAG level 3e1 and are listed in Table 31.8.

Table 31.8. CAG level 3e1: Oxidative stress

2-Phenylphenol	Diuron	

The standard toxicological guidelines do not include as a mandatory requirement studies on oxidative stress. Therefore, more active substances than those listed may cause urinary bladder toxicity by an oxidative stress mode of action.

31.2.4. CAG level 4: Mechanism of action

For a few of the active substances affecting the urinary bladder, a mechanism of action has been proposed. For the remaining substances, no information regarding mechanisms has been found and consequently, these substances cannot be allocated to a CAG level 4.

31.2.4.1. CAG level 4a1a: Increased calcium in urine

Both fluoxastrobin and fosetyl induce calculi in the urinary bladder. For both substances mechanistic studies have shown an increased level of phosphorous in the faeces, a decreased level of phosphorous in the urine and an increased level of calcium in the urine. The two substances differed in that the urinary pH was increased for fluoxastrobin but decreased for fosetyl. For fluoxastrobin it was stated that fluoxastrobin resulted in reduced phosphate absorption in the intestine. A potential phosphate deficiency was counter-regulated by reduced renal excretion of phosphate and renal hyper-excretion of calcium. It is proposed by the DAR that increased calcium excretion in urine, together with an increase in urinary pH, led to calculi formation and following erosive and/or irritative effects of these foreign bodies in the urine, moderate to marked diffuse hyperplasia of the transitional epithelium of the urinary tract with inflammation developed.

For four other of the active substances (2-phenylphenol, chloridazon, oxasulfuron, and sulfosulfuron), which induced calculi, mineralisation was noted in the urinary bladder. Mineralisation in the rat kidney mainly represents calcium salt deposition (Hard et al. 1999). Thus for these substances an increased level of calcium in the urine may also be part of the cause of bladder calculi. However, no mechanistic studies have been performed to confirm or reject that hypothesis.

The active substances identified in mechanistic studies as inducing increased calcium in urine are allocated to CAG level 4a1a and are listed in Table 31.9.

Table 31.9. CAG level 4a1a: Increased calcium in urine

Fluoxastrobin	Fosetyl	

31.2.4.2. Information not considered relevant for CAGs at level 3 and/or 4

According to the DAR, the urinary bladder tumours seen in male rats exposed to high doses of the cyromazine metabolite, melamine, appear to be produced by a non-DNA-reactive mechanism involving epithelial hyperplasia secondary to the presence of melamine-containing bladder stones.

For iprodione the crystals probably consisted of the metabolite 32490 RP.

For thiabendazole the main component of calculi was protein.

The information on the content of bladder stones and crystals for melamine and iprodione is of no relevance for CAGs at level 4 unless other active substances produce the same

metabolites and that is not the case. In order to create a CAG at level 4 for thiabendazole, more mechanistic studies are needed to explain whether protein is important for the formation of the calculi.

2-Phenylphenol (including the sodium salt of orthophenyl phenol):

Despite many mechanistic studies with 2-phenylphenol the mechanism(s) of urinary bladder toxicity is not clear-cut. Calculi were produced in a couple of lifetime studies. However, according to the DAR, the mechanism of tumourigenesis in rats was assumed to be non-genotoxic, probably based on chronic irritation of the epithelium by a combination of high pH, high sodium-ion concentration and/or high concentration of free metabolites at high doses. In the studies there was e.g. a positive correlation between urinary pH and the incidence of hyperplasia of the urinary bladder. The tumourigenic potential of 2-phenylphenol was enhanced by co-administration of sodium bicarbonate as an alkalinising agent while the tumourigenesis by its sodium salt was attenuated by co-administration of ammonium chloride as an acidifier.

The authors of an in vivo micronucleus study from the open literature suggest that both chromosomal alterations and cell proliferation occurring in the rat bladder may contribute to the carcinogenicity of 2-phenylphenol. The DAR does not contain in vivo micronuclei studies.

In addition a study in the open literature has shown that 2-phenylphenol induce oxidative stress in urinary bladder as described in CAG level 3b.

Diuron:

EFSA is concluding that the effects on the bladder can be caused by irritation but there is no clear indication of this in the available documentation.

31.3. Discussion of CAGs for the urinary bladder

Thirty-one active substances were identified to have effects on the urinary bladder and were allocated to CAG level 1 (Table 31.1). Four distinct CAGs at level 2 have been proposed. Information on mode / mechanism of action is available for about half of the active substances. The information is summarised in Appendix AU.

Four distinct CAGs at level 2 have been proposed. All but two of the active substances (lufenuron and phenmedipham) induce hyperplasia of the urinary epithelium and are allocated to CAG level 2a. Hyperplasia of the urinary bladder may be a response to epithelial irritation, which in turn can be induced by urinary crystals, calculi or toxic chemicals. For about half of the active substances the epithelial irritation may be explained by urinary crystals (CAG level 3a2) and/or calculi (CAG level 3a1). As calculi frequently during the course of an experiment are passed by the animals without being detected by the investigators, more active substances than those listed may cause urinary bladder toxicity by a calculi mode of action.

For one of the active substances, 2-phenylphenol, where calculi have been found in a couple of studies, calculi does not seem to be considered part of the mode of action for 2-phenylphenol by the DAR. For most of the active substances, mechanistic studies have not

been performed, and the DAR has not elaborated on the mode or mechanism of action. For fluoxastrobin and fosetyl mechanistic studies have shown an increased level of calcium in the urine (CAG level 4a1a), which may explain the formation of calculi and the subsequent urinary bladder toxicity. For the cyromazine metabolite, melamine, the DAR considers the melamine-containing bladder stones to be the cause of the urinary bladder toxicity. Thus the DARs have no opinion on whether the calculi or crystals may be the cause of urinary bladder toxicity for 12 out of the 16 active substances that induce calculi or crystals. For 3 substances (fluoxastrobin, fosetyl, and the cyromazine metabolite, melamine) the DAR consider the calculi the cause of the urinary bladder toxicity. And for 2-phenylphenol calculi does not seem to be considered part of the mode of action by the DAR.

As cell death (CAG level 2b), inflammation (CAG level 2c) and neoplasms (CAG level 2d) for all but 2 substances probably are part of the same irritative mode of action as for hyperplasia it is not recommend to consider CAG level 2b, CAG level 2c and CAG level 2d for CRA. As oxidative stress (CAG level 3e1) only have been studied for two of the substances (2-phenylphenol and diuron) and many other mechanisms also have been suggested for 2-phenylphenol, it is not recommended to consider CAG level 3e1 for CRA.

31.4. Recommended CAGs for the urinary bladder

The following CAG at level 2 are recommended for CRA for effects on the urinary bladder:

- CAG level 2a: Hypoplasia, see Table 31.2.

32. Database

The results of the evaluations of the available toxicological studies for the allocation of the active pesticide substances into CAGs are presented in the CAPEG database, where the information can be searched. The database is in Microsoft Access version 2003 based on the Excel spreadsheet that was developed for the evaluation of the toxicological studies.

The Excel spreadsheet (and thus the CAPEG database) contains the following entries:

- Substance number
- Active substance (trivial name)
- Category (pesticidal)
- Chemical class
- Chemical name (IUPAC)
- CAS number
- Pesticidal mode/mechanism of action
- ADI (mg/kg bw/d)
- ArfD (mg/kg bw/d)

- AOEL (mg/kg bw/d)
- Target organ/tissue (CAG level 1)
- Phenomenological specific effect (CAG level 2)
- Species
- Strain
- Sex
- Duration
- Route of exposure
- NOAEL (mg/kg bw/d)
- LOAEL (mg/kg bw/d)
- Reference
- Source (DAR, EFSA peer-review, open literature, etc.)
- Remarks (to the outcome of the evaluation)
- Toxicological mode of action (CAG level 3)
- Toxicological mechanism of action (CAG level 4).

The Excel spreadsheet was directly transformed into Microsoft Access 2003 without any further development. The database therefore contains exactly the same entries on the same level as the spreadsheet.

A user manual containing a short introduction to the search functionality of the CAPEG system is attached as Appendix AV.

33. Limitations of approaches and resulting uncertainties

Uncertainty is a consequence of imperfect knowledge and limited data.

In this project the decision to allocate active substances into a certain CAG is primarily dependent on the interpretation of the citations and evaluations of the toxicological studies presented in the individual DARs. There are (at least) two areas that give rise to uncertainty:

- The inherent degree of uncertainties in the measure of effects in the toxicological studies: In many cases, specific endpoints were observed in only one or a few studies, and/or the findings were not consistent across studies, sex and/or species. In this respect, it should be noted that many studies included in the DARs are of an older date and not meeting current requirements.
- The inconsistencies between the various rapporteurs descriptions and interpretations of the same toxicological effect: This may result in one active substance being evaluated as having a given effect while another substance has not, or vica verca. This may either

lead to a substance being wrongly allocated into a given CAG, or on the other hand that the substance is wrongly excluded from a CAG.

34. Further needs for data and research in regard to CRA

Data on mode/mechanism of action are lacking for the vast majority of the different toxicological effects produced by the active pesticide substances included in this assessment. Obviously, such data is needed in order to improve the establishment of CAGs for CRA However, before using many resources to obtain such information a systematic research review on pesticide occurrence and exposure data for Europe should be carried out with the aim of identifying combinations of active pesticide substances that are either realistic candidates for CRA or not.

Conclusions and Recommendations

CONCLUSIONS

Cumulative assessment groups (CAG) for phenomenological effects at CAG level 2 were recommended to be considered for use in cumulative risk assessment (CRA) for the following target organs/tissues:

- Adrenal gland (chapter 7)
- Bone marrow (chapter 8)
- Bones / skeleton (chapter 9)
- Cardiovascular system (chapter 10)
- Eye (chapter 11)
- Gallbladder (chapter 12)
- Haematological system (chapter 14)
- Kidney (chapter 16)
- Liver (chapter 17)
- Muscles (chapter 20)
- Nervous system (chapter 21)
- Parathyroid gland (chapter 23)
- Reproductive system and developmental toxicity (chapter 25)
- Spleen (chapter 28)
- Thyroid gland (chapter 30)
- Urinary bladder (chapter 31)

No CAGs were recommended for CRA the following target organs/tissues:

- Gastrointestinal tract (chapter 13)
- Immune system (chapter 15)
- Lung (chapter 18)
- Lymph node (chapter 19)
- Pancreas (chapter 22)
- Pituitary gland (chapter 24)
- Salivary gland (chapter 26)
- Skin (chapter 27)
- Thymus (chapter 29)

The CAGs at level 2 are established without any knowledge about mode/mechanism of action and do therefore not fulfil the criteria for a cumulative mechanism group that can be expected to exert dose additivity. However, risk managers may wish to use a CAG at level 2 in order to consider whether a realistic cumulative exposure to certain active substances would need to be further investigated.

CAGs at level 3 based on knowledge on the mode of action could only be recommended for the bone marrow, bones / skeleton, eye, haematological system, liver, muscles, nervous system, reproductive and developmental system, and thyroid gland, whereas CAGs at level 4 based on the mechanism of action could only be recommended for some toxicological effects on the eye, liver, nervous system and reproductive and developmental system. These CAGs should provide a reasonable basis for performing CRA.

A searchable database in Microsoft Access was developed containing information on the chemical and pesticidal properties, the acceptable daily intake (ADI) established by the EU for the active substances, and the results of the evaluations of the toxicological studies used to allocate the active substances into the different CAGs, including the NOAELs and LOAELs for these studies (see chapter 32).

RECOMMENDATIONS

A systematic review, based on pesticide occurrence and exposure data for foods in Europe should be carried out with the aim of identifying realistic combinations of active pesticide substances that are relevant candidates for cumulative risk assessments. Data on mode/mechanism of action will then probably be needed for the majority of the toxicological effects produced by the active pesticide substances to be included in such assessments.

Although the CAGs at level 2 are established without any knowledge about mode/mechanism of action and do therefore not fulfil the criteria for a cumulative mechanism group that can be expected to exert dose additivity they can be useful for a preliminary assessment of realistic mixtures of active substances. This in particular, because it can be expected that such an assessment will be conservative in the absence of dose additivity between the compounds.

For such an assessment, it is recommended to use the Reference point index (RfPI) (based on the NOAELs for the compounds in the CAG) as described by the PPR Panel (EFSA 2008). The RfPI is similar to the Point of departure index (PODI) advocated by EFSA (2007) and by WHO (2009) to be used in cumulative risk assessment.

The Reference Point Index (RfPI) represents the sum of the exposures to each pesticide expressed as a fraction of their respective RfPs for the relevant effect (e.g., the dose that causes a 10% effect, BMD_{10}; or the NOAEL). When the RfPI multiplied by a chosen group uncertainty factor (UF) is lower than 1, the combined risk is considered acceptable. An UF of 100 is recommended.

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Appendices

Remark: Appendices in separate file.

Glossary / Abbreviations

GLOSSARY

Active substance	Any substance, including micro-organisms, having general or specific action against harmful organisms or on plants, parts of plants or plant products (Regulation (EC) No 1107/2009)
Adaptive effects	The effect is observed after relative short time of exposure but vanish after prolonged exposure
Agonist	An agonist is a chemical that binds to a receptor of a cell and triggers a response by that cell
Antagonism	Two or more agents in combination have an overall effect which is less than the sum of their individual effects, or: A type of receptor ligand or drug that does not provoke a biological response itself upon binding to a receptor but blocks or dampens agonist-mediated responses
Benchmark doses	The dose of a substance that is expected to result in a prespecified level of effect (Setzer & Kimmel 2003)
CAG level 1	Common assessment group for all compounds exerting a toxicological effect on relevant organ/tissue
CAG level 2	Common assessment group for all compounds showing a common toxic effect on a phenomenological/specific effect basis in a target organ/tissue
CAG level 3	Common assessment group for all compounds possessing the same mode of action
CAG level 4	Common assessment group for all compounds possessing the same mechanism of action.

ChemDraw	Molecule editor developed by CambridgeSoft
Cumulative risk	The combined risks from aggregate exposures to multiple agents or stressors
Cumulative risk assessment	Analysis, characterization, and possible quantification of the combined risks to human health from multiple agents or stressors
Deterministic	An inevitable consequence of antecedent sufficient causes
Dose addition	A mixture of compounds in a dilution acts on the same biological site by the same mechanism/mode of action and differ only in their potencies.
Effect points	Toxicological effects on target organs/tissues.
Hazard Index	A summation of the hazard quotients for all chemicals to which an individual is exposed
Interactions	The combined actions of compounds resulting in a stronger (see synergism) or weaker (see antagonism) effect than would be expected based on the assumption of additivity (see dose additivity)
LD_{50}	The median lethal dose of a substance, or the amount required to kill 50% of a given test population.
Mechanism of action	The specific biochemical interaction through which a substance produces an effect on a living organism or in a biochemical system (IPCS 2009b).
Mechanism of pesticidal action	The specific biochemical interaction through which a substance produces an effect on a target organism
Microsoft Access	A relational database management system from Microsoft that combines the relational Microsoft Jet Database with a graphical user interface and software-development tools.
Mode of action	A biologically plausible sequence of key events leading to an observed effect supported by robust experimental observations and mechanistic data.

	A mode of action describes key cytological and biochemical events – that is, those that are both measurable and necessary to the observed effect – in a logical framework (IPCS 2009b).
Open literature	Peer reviewed literature available through public accessible databases.
Pesticide	Substances or mixture of substances intended for preventing, destroying, repelling or mitigating any pest.
Point of departure	See Benchmark doses (or NOAEL).
Probabilistic	A logical relation between statements such that evidence confirming one confirms the other to some degree
Reference Point Index	The sum of the exposures to each pesticide expressed as a fraction of their respective Reference Points for the relevant effect.
Response addition	A mixture of compounds in a dilution where the compounds do not interfere with each other but they all contribute to a common result.
Simple dissimilar action	See Response addition.
Simple similar action	See Dose-addition
Synergistic effect	Effect arising between two or more substances that produces an effect greater than the sum of their individual effects

ABBREVIATIONS

4-HHP	4-Hydroxyphenyl Pyruvate
4-HPLA	4-Hydroxyphenyl Lactic Acid
4-HPAA	4-Hydroxyphenyl Acetic Acid
AChE	Acetylcholineesterase

ACTH	Adrenocorticotropic Hormone
ADI	Acceptable Daily Intake
AhR	Aryl hydrocarbon Receptor
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AOEL	Acceptable Operator Exposure Level
APTT	Activated Partial Thromboplastin Time
AR	Androgen Receptor
ARfD	Acute Reference Dose
AST	Aspartate Aminotransferase
ATP	Adenosine Triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
BMD	Bone Mineral Density
BSP	Bromosulphophtalein
BW	Body Weight
BWG	Body Weight Gain
CAG	Common Assessment Group
CAPEG	Cumulative Assessment of Pesticides Groups
CAR	Constitutive Androstane Receptor
CAS	Chemical Abstracts Service
CBG	Corticosteroid Binding Globulin
CIRCA	Communication & Information Resource Centre Administrator
CMG	Common Mode/Mechanism Group
CNS	Central Nervous System
CoA	Coenzyme A
COT	Committee on Toxicity of Chemicals in Food, Consumer Products and the Environment
CRA	Cumulative Risk Assessment
cRfD	Chronic Reference Dose
CRH	Corticotropin-Releasing Hormone
CS_2	Carbon disulfide
CV	Cardiovascular

CYP	Cytochrome P450
D1	Type I iodothyronine deiodinase
D2	Type II iodothyronine deiodinase
D3	Type III iodothyronine deiodinase
DAR	Draft Assessment Report
DHT	Dihydrotestosterone
ECCO	European Community Co-Ordination
EC No	European Commission Number
EFSA	European Food Safety Authority
EPA	Environmental Protection Agency
EPCO	EFSA Pesticides Peer review Co-Ordination
ERG	Electroretinographic
EU	European Union
FAO	Food and Agriculture Organization
FC	Food Consumption
FSH	Follicle-stimulating hormone
GABA	Gamma-Aminobutyric acid
GD	Gestation Date
GI	Gastrointestinal Tract
GGT	Gamma Glutamyltransferease
GnRH	Gonadotropin Releasing Hormone
GSH	Glutathione
GSH-Px	Glutathione Peroxidase
Hgb	Haemoglobin
HI	Hazard Index
HPPD	4-Hydroxyphenyl Pyruvic Acid Dioxygenase
Ht	Haematocrit
ILSI	International Life Sciences Institute
IPCS	International Programme on Chemical Safety
IUPAC	International Union of Pure and Applied Chemistry
JMPR	Joint FAO/WHO Meeting on Pesticide Residues

LOAEL	Lowest Observed Adverse Effect Level
LD_{50}	Lethal Dose 50%
LDL	Low Density Lipoprotein
LDH	Lactate Dehydrogenase
LH	Luteinizing Hormone
mAChR	Muscarinic Acetylcholine Receptor
MCH	Mean Corpuscular Haemoglobin
MCHC	Mean Corpuscular Haemoglobin Concentration
MCV	Mean Corpuscular Volume
MOA	Mode of Action
MRL	Maximum Residue Level
mRNA	Messenger Ribonucleic Acid
nAChR	Nicotinic Acethylcholine Receptor
NOAEL	No Observed Adverse Effect Level
OATP	Organic Anion-Transporting polypeptide
OCT	Ornithine Carbamyltransferase
OECD	Organisation for Economic Co-operation and Development
OPIDN	Organophosphate-Induced Delayed Neuropathy
PND	Postnatal Day
PNS	Peripheral Nervous System
PODI	Point Of Departure Index
PPARα	Peroxisome Proliferator Activated Receptor alpha
PPDB	Pesticide Properties Database
PPR	Plant Protection Products and their Residues
PT	Prothrombin Time
PU	Propylenurea
PXR	Pregnane X receptor
RBC	Red Blood Cell
RfP	Reference Point
RfPI	Reference Point Index
RMS	Rapporteur Member States

ROS	Reactive Oxygen Species
RPF	Relative Potency Factor
rT_3	Reverse T_3
SCLP	Straight Chain Lepidoptera Pheromones
SCP	Scientific Committee on Plants
SDH	Sorbitol Dehydrogenase
SMILES	Simplified Molecular Input Line Entry System
SOD	Superoxide dismutase
StAR protein	Steroidogenic Acute Regulatory protein
SULT	Sulphotransferases
T_2	Di-iodothyronine
T_3	Triiodothyronine
T_4	Thyroxine
TAT	Tyrosine Transaminase Enzyme
TBG	Thyroxine-Binding Globulin
TPO	Thyroid Peroxidase
TRH	Thyrotropin-Releasing Hormone
TSH	Thyroid-Stimulating Hormone
TTR	Transthyretin
UDPGT	Uridine Diphosphate Glucuronyl-Transferases
UF	Uncertainty Factor
U.S. EPA	United States Environmental Protection Agency
WBC	White Blood Cells
WHO	World Health Organization